

The N-end rule pathway and regulation by proteolysis

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Running title: The N-end rule pathway.

Keywords: / ubiquitin / N-end rule / N-degron / proteolysis / arginylation / leucylation /

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Manuscript information:

Abstract: 250 words.

Main text (*excluding Abstract, legends and references*): 22,660 words.

Number of references: 341. Number of figures: 15.

Abstract

The N-end rule relates the regulation of the *in vivo* half-life of a protein to the identity of its N-terminal residue. Degradation signals (degrons) that are targeted by the N-end rule pathway include a set called N-degrons. The main determinant of an N-degron is a destabilizing N-terminal residue of a protein. In eukaryotes, the N-end rule pathway is a part of the ubiquitin system and consists of two branches, the Ac/N-end rule and the Arg/N-end rule pathways. The Ac/N-end rule pathway targets proteins containing N^α-terminally acetylated (Nt-acetylated) residues. The Arg/N-end rule pathway recognizes unacetylated N-terminal residues and involves N-terminal arginylation. Together, these branches target for degradation a majority of cellular proteins. For example, more than 80% of human proteins are cotranslationally Nt-acetylated. Thus most proteins harbor a specific degradation signal, termed ^{Ac}N-degron, from the moment of their birth. Specific N-end rule pathways are also present in prokaryotes and in mitochondria. Enzymes that produce N-degrons include methionine-aminopeptidases, caspases, calpains, Nt-acetylases, Nt-amidases, arginyl-transferases and leucyl-transferases. Regulated degradation of specific proteins by the N-end rule pathway mediates a legion of physiological functions, including the sensing of heme, oxygen, and nitric oxide; selective elimination of misfolded proteins; the regulation of DNA repair, segregation and condensation; the signaling by G proteins; the regulation of peptide import, fat metabolism, viral and bacterial infections, apoptosis, meiosis, spermatogenesis, neurogenesis, and cardiovascular development; and the functioning of adult organs, including the pancreas and the brain. Discovered 25 years ago, this pathway continues to be a fount of biological insights.

1. Glossary of terms

- ‘*Sequelog*’ and ‘*spalog*’ denote, respectively, a sequence that is similar, to a specified extent, to another sequence, and a three-dimensional (3D) structure that is similar, to a specified extent, to another 3D structure¹. Derivatives of these terms include ‘sequelogenous’ and ‘sequelogy’ (sequence similarity); ‘spalogous’ and ‘spalogy’ (spatial similarity). In addition to their usefulness as separate terms for sequence and spatial similarities, the rigor-conferring advantage of ‘sequelog’ and ‘spalog’ is their *evolutionary neutrality*, in contrast to interpretation-laden terms such as ‘homolog’, ‘ortholog’ and ‘paralog’. The latter terms are compatible with the sequelog/spalog terminology and can be used to convey understanding about functions and common descent, if this (additional) information is available¹.

- *Ubiquitin* (Ub): a highly conserved 76-residue eukaryotic protein that can be enzymatically conjugated to other proteins, thereby marking them for processive degradation or other metabolic fates.

- *N-end rule*: it relates the regulation of the *in vivo* half-life of a protein to the identity of its N-terminal residue.

- *N-end rule pathway*: a proteolytic pathway whose physiological targets include proteins with destabilizing N-terminal residues.

- *Degron*: a degradation signal².

- *N-degion*: one class of degradation signals that can be targeted by an N-end rule pathway.

The main determinant of an N-degion is either a modified or unmodified destabilizing N-terminal residue of a protein.

- *Pro-N-degion*: precursor of N-degion. A pro-N-degion is a specific sequence or conformational determinant of a polypeptide chain that can be cleaved or otherwise modified to produce a destabilizing N-terminal residue. This definition of a pro-N-degion implies that other determinants of an N-degion, e.g., a ‘targetable’ internal Lys residue of a substrate, are in place as well.

- *N-recognin*: recognition component of an N-end rule pathway that recognizes (binds to) specific N-degions.

- *Nd^p residue*: a primary (‘p’) destabilizing N-terminal residue, i.e., an unmodified N-terminal residue that is directly recognized by an N-recognin.

- *Nd^s residue*: a secondary ('s') destabilizing N-terminal residue, i.e., a residue whose destabilizing activity requires a specific preliminary modification, such as, for example, N-terminal arginylation (Nt-arginylation).
- *Nd^t residue*: a tertiary ('t') destabilizing N-terminal residue, i.e., a residue whose destabilizing activity requires two preliminary modifications.
- *Arg/N-end rule pathway*: a branch of the eukaryotic N-end rule pathway that involves the Nt-arginylation of protein substrates and also the targeting of unmodified bulky hydrophobic and basic N-terminal residues by an N-recognin E3 ubiquitin ligase.
- *Ac/N-end rule pathway*: a branch of the eukaryotic N-end rule pathway that may also be present in archaeal prokaryotes. The Ac/N-end rule pathway involves the N^α-terminal acetylation (Nt-acetylation) of nascent proteins whose N-termini bear either Met or the small uncharged residues Ala, Val, Ser, Thr or Cys. These residues become N-terminal after the cotranslational removal of Met by Met-aminopeptidases. Nt-acetylated proteins are targeted for degradation by the Ac/N-end rule pathway³.
- *Leu/N-end rule pathway*: a bacterial N-end rule pathway that involves Nt-leucylation of protein substrates by specific L-transferases and also the targeting of bulky hydrophobic N-terminal residues by the ClpS N-recognin, an adaptor protein that delivers bacterial N-end rule substrates to the ClpAP protease.

2. Introduction

The lifespans of protein molecules in a cell range from less than a minute to many days. Among the functions of intracellular proteolysis are the elimination of misfolded or otherwise abnormal proteins, the maintenance of amino acid pools in cells affected by stresses such as starvation, and the generation of protein fragments that act as hormones, antigens, or other effectors. One major role of proteolytic pathways is the selective destruction of regulatory proteins whose concentrations must vary with time and alterations in the state of a cell. A short *in vivo* half-life of a protein provides a way to generate its spatial gradient and to rapidly adjust its concentration or subunit composition through changes in the rate of its degradation. Proteolysis can also act to activate protein molecules and specific circuits, by removing an autoinhibitory protein domain or by selectively destroying an inhibitory subunit of a protein complex. The regulated (and processive) degradation of intracellular proteins is carried out largely by the ubiquitin-proteasome system (Ub

system) (Fig. 1A), in conjunction with molecular chaperones, autophagy and lysosomal proteolysis. Chaperones mediate *in vivo* protein folding and the assembly/disassembly of protein complexes. A meta-system that includes the Ub system and chaperones determines the time-dependent probability, for each protein, of being either in its 'normal' (functional) state, or targeted for degradation, or perturbed in ways (including aggregation) that may or may not lead to degradation. Other contributors to intracellular proteolysis include cytosolic and nuclear proteases such as caspases and calpains. These and other nonprocessive proteases can function as 'upstream' components of the Ub system, producing protein fragments that are often targeted and degraded to short peptides by Ub-mediated pathways. Proteins that are damaged, misfolded or otherwise abnormal are usually short-lived *in vivo*, with significant exceptions that include a subset of perturbed proteins (and/or their aggregates) that are harmful but cannot be efficaciously repaired or removed. The resulting proteotoxicity underlies both aging and specific diseases, including neurodegeneration.

The N-end rule relates the regulation of the *in vivo* half-life of a protein to the identity of its N-terminal residue. The 1986 discovery of the N-end rule pathway identified the first specific pathway of the Ub system⁴⁻⁶. It was also the discovery of the first primary degradation signals (degrons²) in short-lived proteins⁴. Ub, a 76-residue protein, is a 'secondary' degron in that Ub is conjugated to proteins that contain primary degradation signals. For accounts of the early history of the Ub field, see refs. 6-8. Over the last quarter century, the understanding of the N-end rule pathway has advanced to such an extent that a comprehensive review of the pathway has to be an uncommonly large paper.

3. Overview of the N-end rule pathway

N-terminal degradation signals of the N-end rule pathway are called N-degrons. The main determinant of an N-degron is a destabilizing N-terminal residue of a protein^{3-6,9-44} (Figs. 2-5). In eukaryotes, the N-end rule pathway is a part of the Ub system, which mediates selective protein turnover through the conjugation of Ub to specific proteins, thereby marking them for degradation by the 26S proteasome, a multisubunit ATP-dependent protease^{6,45-65} (Fig. 1A). Prokaryotes, i.e., bacteria and archaea, contain Ub-like proteolytic pathways but lack the *bona fide* Ub system⁶⁶⁻⁷⁰. Nevertheless, prokaryotes contain specific versions of the N-end rule pathway that do not involve ubiquitylation^{5,6,14-16,37,71-78} (Fig. 5).

Recognition components of the N-end rule pathway are called N-recognins⁵. In bacteria, the 12-kDa ClpS, identified as an N-recognin by the Bukau laboratory⁷², binds to N-degrons of N-end rule substrates and delivers them to the ATP-dependent ClpAP protease^{16,72,74,76-82} (Fig. 5 and Fig. 6B-D). In eukaryotes, N-recognins are E3 Ub ligases that bind to specific N-degrons^{3,5,6,22,30,38,39,43,83-85} (Fig. 7 and Fig. 8). A complex of an E3 N-recognin and its cognate E2 Ub-conjugating enzyme polyubiquitylates N-end rule substrates at their internal Lys residues, thereby targeting these proteins for degradation by the 26S proteasome^{9,11} (Fig. 2 and Fig. 3). The term 'Ub ligase' denotes either an E2-E3 complex or its E3 component^{6,54,57,62}. In eukaryotes, the N-end rule pathway comprises two major branches, one of which is termed the Arg/N-end rule pathway. This branch involves the N-terminal arginylation (Nt-arginylation) of protein substrates and also the targeting of specific unmodified N-terminal residues by E3 N-recognins (Fig. 2A and Fig. 3). The other branch is termed the Ac/N-end rule pathway³. It involves the cotranslational N^α-terminal acetylation (Nt-acetylation) of nascent proteins⁸⁶⁻⁹⁵ whose N-termini bear either Met or the small uncharged residues Ala, Val, Ser, Thr or Cys. These residues become N-terminal after the cotranslational removal of N-terminal Met by Met-aminopeptidases⁹⁶⁻¹⁰¹ (Fig. 1C, D). Nt-acetylated proteins are targeted for regulated degradation by the Ac/N-end rule pathway³ (Fig. 2B).

The Nt-acetylated Met, Ala, Val, Ser, Thr and Cys residues of newly formed proteins comprise a specific class of N-degrons, termed ^{Ac}N-degrons³ (Fig. 2B and Fig. 4A). The cotranslational Nt-acetylation of nascent proteins^{87-95,102} (Fig. 1C) is both enzymatically and functionally distinct from the largely posttranslational acetylation of internal residues in many proteins^{103,104}. Nt-acetylation and internal acetylation are carried out by (mostly) nonoverlapping sets of specific acetylases. In addition, Nt-acetylation is apparently irreversible. No Nt-deacetylases have been identified, in contrast to a dynamic internal acetylation/deacetylation, with specific deacetylases removing internally conjugated acetyl groups (ref. 104 and refs. therein). As described below, the proteolytic function of Nt-acetylation (Fig 2B) is likely to be relevant to more than 80% of the entire proteome, i.e., to thousands of Nt-acetylated proteins³. In contrast, either an identified or inferred necessity of Nt-acetylation for other (nonproteolytic) functions involves, at present, only ~10 Nt-acetylated proteins (refs. 92,105-110 and refs. therein).

Apart from expanding the N-end rule and its functions, the 2010 discovery of the Ac/N-end rule pathway³ has also revealed the main physiological roles of two classes of enzymes, Nt-acetylases and Met-aminopeptidases. Specifically, Nt-acetylases produce ^{Ac}N-degrons while the 'upstream'

Met-aminopeptidases, by cleaving off the N-terminal Met residue, make these degradation signals possible, all of them save for those ^{Ac}N-degrons that contain the Nt-acetylated N-terminal Met (Fig. 1C, D and Fig. 2B). Nt-acetylases and Met-aminopeptidases are essential and universally present enzymes⁸⁶⁻¹⁰¹ whose physiological functions had been largely unknown. These enzymes are now specific components of the Ac/N-end rule pathway³ (Fig. 2B).

N-terminal Arg, Lys, His, Leu, Phe, Tyr, Trp, Ile, Asp, Glu, Asn, Gln, and Cys comprise the main determinants of N-degrons in the Arg/N-end rule pathway (Fig. 2A and Fig. 3). Among these N-degrons, the unmodified basic (Arg, Lys, His) and bulky hydrophobic (Leu, Phe, Tyr, Trp, Ile) N-terminal residues are recognized directly by cognate E3 N-recognins (Fig. 2A, Fig. 3, Fig. 7 and Fig. 8). These E3s contain highly spalogous (spatially similar¹) ~80-residue regions called UBR domains or type-1 binding sites⁸³⁻⁸⁵. Folded around three zinc ions, a UBR domain binds to N-terminal Arg, Lys or His, the type-1 primary destabilizing residues of N-end rule substrates (Fig. 3 and Fig. 8). Another (usually adjacent) region of UBR-type N-recognins, called the type-2 binding site, recognizes N-terminal Leu, Phe, Tyr, Trp or Ile, which are called the type-2 primary destabilizing residues (Fig. 7A). Together, the directly recognized primary destabilizing N-terminal Arg, Lys, His, Leu, Phe, Tyr, Trp and Ile are denoted as Nd^P residues (*N*, N-terminal; *d*, destabilizing; *p*, primary)^{6,43,83-85}. In contrast to these residues, the N-terminal Asp, Glu, Asn, Gln and Cys function as destabilizing residues through their preliminary modifications. One of these modifications is Nt-arginylation. N-terminal Arg is an Nd^P residue, i.e., it can be recognized by E3 N-recognins of the Arg/N-end rule pathway (Fig. 2A and Fig. 3). Arg-tRNA-protein transferase (R-transferase) conjugates Arg to N-terminal Asp, Glu or oxidized Cys of proteins or short peptides, with Arg-tRNA as the cosubstrate and the donor of Arg. R-transferases are encoded by *Ate1* and its sequelogs from yeast to mammals but are absent from examined prokaryotes^{32,35,36,111-114} (Fig. 9). In contrast to N-terminal Asp, Glu and oxidized Cys, the N-terminal Asn and Gln residues cannot be arginylated by R-transferase. However, the Arg/N-end rule pathway contains specific N-terminal amidases (Nt-amidases) that convert N-terminal Asn and Gln to Asp and Glu, respectively, followed by their Nt-arginylation^{23,25,42,115-117} (Fig. 2A and Fig. 3).

N-terminal Cys residues can be Nt-acetylated (in proteins that contain the initially present N-terminal Met-Cys sequence) after the cotranslational removal of N-terminal Met by Met-aminopeptidases (Fig. 1C, D). The same is true for other second-position (penultimate) small residues such as Ala, Val, Ser or Thr. Nt-acetylation of Cys produces an ^{Ac}N-degron of the Ac/N-end

rule pathway and thereby precludes the (alternative) participation of N-terminal Cys in the Arg/N-end rule pathway³ (Fig. 2B; cf. Fig. 3). However, some sequence contexts, e.g., a basic residue at position 2, inhibit the Nt-acetylation of N-terminal Cys and other N-terminal residues. The mammalian proteins Rgs4, Rgs5 and Rgs16 are one example of this inhibition. They bind to G α subunits of specific G proteins and increase the intrinsic GTPase activity of G α , thereby down-regulating the signaling by these G proteins. The N-terminal Cys residue of Rgs4, Rgs5 and Rgs16 is followed by a basic residue (Fig. 10), hence the absence of Nt-acetylation of these RGS proteins^{32,33,118}. (Whereas in the yeast *Saccharomyces cerevisiae* a basic residue at position 2 suffices to block Nt-acetylation, some proteins with position-2 basic residues can be Nt-acetylated in mammalian cells⁹¹.)

In addition, conditional cleavages of cellular proteins by nonprocessive proteases such as caspases or calpains can also produce C-terminal fragments that bear unmodified N-terminal Cys residues. If the protein's N-terminal Cys can be oxidized through (apparently nonenzymatic) reactions that require both nitric oxide (NO) and oxygen, and if these compounds are present in a cell at sufficient levels, the resulting N-terminal Cys-sulfinate or Cys-sulfonate (but not the original Cys) can be Nt-arginylated by the Ate1 R-transferase^{32,33}. The necessity of NO and oxygen for the destabilizing activity of N-terminal Cys makes the Arg/N-end rule pathway a sensor of both NO and oxygen (Section 9.4). In sum, depending on specific protein substrates and *in vivo* conditions such as the presence of NO, the N-terminal Cys of a protein can function either as an NO/O₂-mediated N-degron of the Arg/N-end rule pathway or, alternatively, as an ^{Ac}N-degron of the Ac/N-end rule pathway^{3,32,33} (Fig. 2B and Fig. 3).

N-terminal Asp, Glu, Asn, Gln and Cys that are targeted by the Arg/N-end rule pathway are termed 'secondary' (Nd^s) or 'tertiary' (Nd^t) destabilizing residues, depending on the number of specific modifications (arginylation of Asp and Glu; deamidation/arginylation of Asn and Gln; oxidation/arginylation of Cys) that precede the targeting and polyubiquitylation, by N-recognins, of Nt-arginylated Arg/N-end rule substrates^{15,32,33,36,41,42}. Analogously, the N-terminal Met, Ala, Val, Ser, Thr and Cys residues that become ^{Ac}N-degrons after the Nt-acetylation of these residues, are classed as Nd^s (secondary destabilizing N-terminal) residues, because they must be Nt-acetylated before their targeting by N-recognins of the Ac/N-end rule pathway³ (Fig. 2B).

Together, the Arg/N-end rule and Ac/N-end rule pathways target a majority of cellular proteins for regulated degradation. For example, more than 80% of human proteins are

cotranslationally Nt-acetylated by a family of ribosome-associated Nt-acetylases that act after Met-aminopeptidases, which are also bound to the ribosomes^{89,91,92,95} (Fig. 2B). Thus, remarkably, most proteins harbor a specific degradation signal (^{Ac}N-degron) from the moment of their birth³. Posttranslational Nt-acetylation of proteins can occur as well (J.-H. Oh and A.V., unpubl. data), presumably because there is a significant pool of Nt-acetylases that are not bound to ribosomes.

In sum, N-degrons of the Ac/N-end rule and Arg/N-end rule pathways can be produced either cotranslationally or posttranslationally (and conditionally), by enzymes that include caspases, calpains, separases, other nonprocessive proteases, Nt-acetylases, Nt-amidases, and R-transferases. These enzymes function as upstream components of the N-end rule pathway, preparing its substrates for targeting and polyubiquitylation by N-recognins^{6,15,16} (Fig. 2 and Fig. 3). In contrast to Nt-arginylation in eukaryotes, N-end rule substrates that bear Nd^s residues in bacteria are Nt-leucylated by the Aat L/F-transferase or the Bpt L-transferase, which conjugate (largely) Leu, an Nd^p residue, to N-terminal Nd^s residues of bacterial proteins, prior to their recognition by the ClpS N-recognin^{16,37} (Fig. 5).

Physiological functions of the N-end rule pathway are strikingly broad and continue to be discovered. Regulated degradation of proteins by the eukaryotic Arg/N-end rule pathway (Figs. 2A and 3) mediates the sensing of heme, NO, oxygen and short peptides; the selective elimination of misfolded proteins; the regulation of DNA repair (through degradation of Mgt1, a DNA repair protein); the cohesion/segregation of chromosomes (through degradation of a subunit of cohesin); the signaling by transmembrane receptors (through degradation of the G-protein regulators Rgs4, Rgs5 and Rgs16); the control of peptide import (through degradation of Cup9, the import's transcriptional repressor); the regulation of apoptosis, meiosis, viral and bacterial infections, fat metabolism, cell migration, actin filaments, cardiovascular development, spermatogenesis, neurogenesis, and memory; the functioning of adult organs, including the brain, muscle, testis and pancreas; and the regulation of leaf and shoot development, leaf senescence and seed germination in plants (refs. 3,6,15,16,18,26,32,34,36,39-42,113,119-136 and refs. therein). Mutations in UBR1, an E3 N-recognin of the human Arg/N-end rule pathway, cause Johanson-Blizzard syndrome (JBS). It comprises physical malformations, insufficiency and inflammation of the exocrine pancreas, and frequent mental retardation as well as deafness³⁴. Remarkably, an N-recognin such as mammalian UBR2 can also function to *protect* specific proteins from degradation¹³⁷. The recently discovered Ac/N-end rule pathway (Fig. 2B) is likely to mediate, among other things, protein quality control, the

regulation of *in vivo* stoichiometry of proteins that form multisubunit complexes, and the degradation of long-lived proteins³. Physiological roles of the bacterial (*E. coli*) N-end rule pathway include the regulated degradation of the Dps nucleoid-condensing protein and the YgjG putrescine aminotransferase^{76,77}.

4. Terminology and definitions

The terms used in this review are defined above and in Glossary (Section 1). The notations ‘Arg/N-end rule pathway’ and ‘Ac/N-end rule pathway’ (Fig. 2 and Fig. 3) should be applicable to any eukaryote, as they bring up a key modification, either Nt-arginylation (‘Arg’) or Nt-acetylation (‘Ac’) but do not invoke specific genes or proteins. In this terminology, the bacterial N-end rule pathway is called the Leu/N-end rule pathway, given its similarity (despite the absence of ubiquitylation) to the eukaryotic Arg/N-end rule pathway, with Nt-leucylation in bacteria versus Nt-arginylation in eukaryotes (Fig. 5).

As an experimentally observed but formal (non-mechanistic) relation between the regulation of the *in vivo* half-life of a protein and the identity of its N-terminal residue, the N-end rule does not place constraints on the nature of processing steps (e.g., proteolytic cleavages) or specific enzymes that produce N-degrons and implement the N-end rule pathway^{5,6,15,16}. An N-degron is classed as such if an unmodified or covalently modified N-terminal residue of a protein is an essential determinant of that protein’s degradation signal. (A protein may contain, and often does, other degrons as well.) This function-based definition does not specify molecular devices that produce, recognize or regulate N-degrons. It is also compatible with any route through which a destabilizing residue becomes N-terminal in a polypeptide. In sum, a feature that suffices to demarcate a processive proteolytic pathway as a branch of the N-end rule pathway is its ability to target specific N-terminal residues, unmodified or covalently modified.

Substrate-binding sites of an E3 N-recognin that targets N-degrons of protein substrates are apparently always accessible, whereas other sites of the same E3, the ones that target internal degrons of other protein substrates, can be autoinhibited. This autoinhibition can be allosterically reversed by ligands such as short peptides that bind to the sites of N-recognin that target N-degrons^{27,29,38,138}. Given these functionally important connections among different degron-recognizing sites of N-recognins, an intracellular protein is classed as a substrate of the N-end rule pathway if it is targeted by an N-recognin, irrespective of whether the targeting involves an internal degron of the

protein or an N-degron⁶. This hardware-centric (N-recognin-based) definition of substrates of the N-end rule pathway bypasses the semantically intractable issue of multiple binding sites in N-recognins.

5. Substrates of the N-end rule pathway

An N-degron can be produced from a pro-N-degron (precursor of N-degron) through a cotranslational or posttranslational proteolytic cleavage. Ribosome-associated Met-aminopeptidases cleave off the Met residue from the N-terminus of a nascent protein if the residue at position 2, to become N-terminal after cleavage, has a small enough side chain^{100,101} (Fig. 1D). Consequently, of the 13 residues that are destabilizing in the mammalian Arg/N-end rule pathway, only Cys can be made N-terminal by Met-aminopeptidases (Fig. 1D and Fig. 3). (Any destabilizing residue, including Cys, can be made N-terminal through posttranslational cleavages of proteins by other nonprocessive proteases^{6,28}.) In contrast to larger residues at position 2, the second-position Ala, Val, Ser, Thr or Cys can be made N-terminal by Met-aminopeptidases³ (Fig. 1D). These residues, which are usually Nt-acetylated, are the secondary destabilizing (Nd^s) residues of the Ac/N-end rule pathway³ (Fig. 2B). The initial N-terminal Met of a nascent protein is also an Nd^s residue of the Ac/N-end rule pathway, the only such residue that does not require a preliminary proteolytic cleavage to form an ^{Ac}N-degron. If N-terminal Met is followed by a bulky residue, this Met is not cleaved off, and is usually Nt-acetylated³ (Fig. 1C, D and Fig. 2B).

More than 80% of mammalian proteins are cotranslationally Nt-acetylated⁹¹⁻⁹³. About 20 Nt-acetylated proteins, largely in *S. cerevisiae* but also in mammalian cells and chosen nearly at random, have been examined, thus far, for the presence ^{Ac}N-degrons, using methods that included pulse-chase and cycloheximide-chase assays, as well as genetic techniques. Nearly every one of the tested Nt-acetylated proteins was found to contain an ^{Ac}N-degron (ref. 3; A. Shemorry, C.-S. Hwang, B. Wadas and A.V., unpubl. data). Given these results and the pervasiveness of Nt-acetylation, our current premise is that most cellular proteins can become substrates of the Ac/N-end rule pathway, either during their synthesis, or immediately afterwards, or significantly later (Fig. 2B). In contrast, the Arg/N-end rule pathway appears to target fewer substrates (but still hundreds of them; see below), in part because most N-degrons of this pathway are produced posttranslationally, through cleavages by proteases other than Met-aminopeptidases. That is so because second-position residues that are destabilizing in the Arg/N-end rule pathway are too large to be made N-terminal by

Met-aminopeptidases (Fig. 1D, Fig. 2A and Fig. 3). The sole exception is Cys (Fig. 4A). Specifically, the N-degrons of the mammalian Rgs4, Rgs5 and Rgs16 proteins (Sections 3 and 9.4) would be expected to form cotranslationally or nearly so in the presence of NO and other conditions that are conducive to oxidation of N-terminal Cys (Fig. 3 and Fig. 10). N-degrons also form cotranslationally in engineered N-end rule substrates that are expressed *in vivo* as Ub-X-protein fusions (in which X is a destabilizing residue), because deubiquitylases cotranslationally remove the fusion's Ub moiety^{9,24,139}. In addition, UBR-type E3 N-recognins of the Arg/N-end rule pathway recognize not only N-degrons but also internal (non-N-terminal) degradation signals (Fig. 2A and Fig. 3). Proteins that lack N-degrons but contain these internal degrons comprise yet another set of substrates of the Arg/N-end rule pathway. Only a few (out of probably many) such substrates have been identified so far (Section 9.4).

Physiological substrates of the eukaryotic Arg/N-end rule pathway include the *Drosophila melanogaster* DIAP1 regulator of apoptosis^{140,141}; the mammalian Rgs4, Rgs5, and Rgs16 regulators of G proteins^{32,33,118}; the C-terminal fragments of the Scc1/Rad21 cohesin subunit that are produced by separase in eukaryotes from yeast to mammals (ref. 28; J. Zhou, D. Pati and A.V., unpubl. data); the human immunodeficiency virus-1 (HIV-1) integrase^{132,133}; and the *Listeria monocytogenes* listeriolysin (Lys-LLO), which is secreted by this bacterium into the cytosol of infected mammalian cells¹²⁴ (Fig. 10 and Section 9.4). In addition, the *S. cerevisiae* Mgt1 DNA repair protein and the Cup9 transcriptional repressor are targeted by the Arg/N-end rule pathway through their internal degrons (Section 9.4). Figure 10 and a brief description below cite the currently known substrates of the Arg/N-end rule pathway (other than Mgt1 and Cup9) and a few putative (unverified) N-end rule substrates that are a part of a much larger set of such substrates (A.V., unpubl. data). For some of these protein fragments, published evidence suggests their metabolic instability; other fragments have not been examined in this regard.

1) C-terminal fragment of HEF1 (a focal adhesion-associated docking protein) that bears N-terminal Tyr (an Nd^P residue) and is produced by caspases not only during apoptosis but during normal mitosis as well^{142,143} (Fig. 10).

2) C-terminal fragment of the MET tyrosine kinase, a transmembrane receptor of HGF/SF, a hepatocyte growth factor-scatter factor. This fragment of MET is produced by caspases (if MET is not bound to HGF/SF) and bears N-terminal Tyr, an Nd^P residue¹⁴⁴⁻¹⁴⁷. MET is a member of the family of more than 10 mammalian 'dependence' receptors (DRs). These transmembrane receptors

are usually not related by sequence or structure but are functionally analogous because of their ability to mediate two opposite physiological outcomes. In the presence of its cognate ligand, a DR receptor activates signaling pathways that mediate cell survival, migration and differentiation. However, in the absence of a cognate ligand, a DR receptor acquires an 'opposite' activity, i.e., it produces an apoptotic signal, often through the formation, by caspases or other nonprocessive proteases, of a proapoptotic C-terminal cytosolic fragment(s) that functions in the cytosol and/or the nucleus^{146,147}.

3) C-terminal fragment of the MYC oncoprotein, termed MYC-nick¹⁴⁸, that is produced by calpain(s), bears N-terminal Arg (an Nd^P residue), and exhibits physiological activities that are different from those of full-length MYC.

4) C-terminal fragment of the ETK/BMX tyrosine protein kinase that is produced by caspases, bears N-terminal Trp (an Nd^P residue), and is proapoptotic¹⁴⁹.

5) C-terminal fragment of the transmembrane EPHA4 'dependence' receptor (item 2 above^{146,147}) that is produced by caspases (if EPHA4 is not bound to its ligand EPHB3) and bears N-terminal Asp, an Nd^S residue¹⁵⁰.

6) C-terminal fragment of the mouse Cdc42 GTP-binding protein that is produced by caspases and bears N-terminal Asp, an Nd^S residue¹⁵¹.

7) C-terminal fragment of the MDM2 E3 Ub ligase (whose targets include p53) that is produced by caspases and bears N-terminal Cys, an Nd^T residue¹⁵².

8) C-terminal fragment of the protein kinase C δ (PKC δ) that is produced by caspases, bears N-terminal Asn (an Nd^T residue), and is proapoptotic, in contrast to the full-length PKC δ kinase^{153,154}.

9) C-terminal fragment of the protein kinase C θ (PKC θ) that is produced by caspases, bears N-terminal Lys (an Nd^P residue), and is proapoptotic, in contrast to the full-length PKC θ kinase^{153,155} (Fig. 10).

Many more substrates of the Arg/N-end rule pathway that contain N-degrons are likely to exist in mammals, but they remain either putative or unknown, given the logistics and uncertainties of current proteome-scale assays, and also because of insufficient knowledge about nonprocessive (and conditional) cleavages of intracellular proteins that yield *in vivo* N-end rule substrates. The expected multitude of such substrates stems from the existence of nonprocessive proteases that function, in particular, in the nucleus and/or cytosol and are known or expected to produce C-terminal fragments of specific proteins that bear destabilizing N-terminal residues of the Arg/N-end rule pathway. These proteases include Met-aminopeptidases⁹⁶⁻¹⁰¹, caspases¹⁵⁶⁻¹⁶⁰, calpains, separase^{161,162}, taspase¹⁶³,

MALT1 protease¹⁶⁴, γ -secretase¹⁶⁵, proteinase-3 (PR3)¹⁶⁶, and viral proteases¹³¹⁻¹³³. It should be emphasized that caspases cleave specific intracellular proteins not only in settings that lead to apoptotic cell death but also in pathways of cell differentiation that do not result in cell death^{158,159}. In addition, if the cleavage of a protein by a caspase produces a proapoptotic C-terminal fragment, it being a short-lived N-end rule substrate would counteract apoptosis and thus 'buffer' a cell against toxicity of caspases that become active owing to a significant level of noise in caspase-activation circuits (Section 9.4).

In addition to the substrates cited in Fig. 10, several other likely *in vivo* substrates of the Ate1 R-transferase (Fig. 2A and Fig. 3) include protein disulfide isomerase (PDI), glucose-regulated protein 78 (Grp78), β -actin, γ -actin, and calreticulin^{35,127,128,130,135,136}. Although the Ate1 R-transferase and the rest of the Arg/N-end rule pathway are apparently confined to the cytosol and the nucleus, and although calreticulin, Grp78 and PDI are present largely in the lumen of the endoplasmic reticulum (ER), a variety of evidence suggests that these proteins (lacking their cleaved-off signal sequences and bearing N-terminal Nd^s residues) are also present in the cytosol and other non-ER compartments, where they may become R-transferase substrates^{35,136}. Partial Nt-arginylation of apparently long-lived proteins such as β -actin and calreticulin^{128,135,136} suggests that Nt-arginylation may have nonproteolytic functions as well.

Hamilton *et al.*¹⁶⁷ characterized G_o/G_i heterotrimeric G proteins purified from bovine brains. The bulk of the G γ 2 subunit of the G_o protein had the expected N-terminal sequence Ac-ASNNTASIA, produced through the removal of N-terminal Met by Met-aminopeptidases and the (presumably) cotranslational Nt-acetylation of N-terminal Ala (Sections 3 and 8, and Fig. 2B). However, a minor but significant fraction of the G γ 2 subunit of G_o protein had the N-terminal sequence RDTASIA. Such a sequence would be produced from ASNNTASIA via the removal (through a single cleavage or sequential proteolysis) of the first 3 residues (after initial Met) by an unknown protease, deamidation of the resulting N-terminal Asn by the Ntan1 Nt^N-amidase (Fig. 3 and Section 9.2) and Nt-arginylation of the resulting N-terminal Asp by the Ate1 R-transferase¹⁶⁷. The Arg-G γ 2 protein produced from engineered Ub-Arg-G γ 2 using the Ub fusion technique (Fig. 1B) was a short-lived N-end rule substrate in reticulocyte extract¹⁶⁷. It remains to be determined whether Arg-G γ 2 is a physiological N-end rule substrate, because the still unexcluded possibility is that Arg-G γ 2 might be produced through *in vitro* proteolysis, Nt-deamidation and Nt-arginylation in crude extracts during purification of G proteins¹⁶⁷.

The currently known physiological substrates of the bacterial Leu/N-end rule pathway (Fig. 5) are discussed in Section 10.

6. Structure and targeting of N-degrons

Mechanistic aspects of the N-end rule pathway that are critical for its functions include the regulation of E3 N-recognins, for example through a specific phosphorylation cascade³⁹ (Fig. 7), and also through changes in the activity/accessibility of degrons in N-end rule substrates^{27,29,138}. A key mechanistic capability of the N-end rule pathway is its subunit selectivity^{13,168}, i.e., the ability to selectively target and destroy a subunit of a protein complex while sparing the rest of it (Fig. 11A-D). Examples of subunit-selective protein remodeling by the Arg/N-end rule pathway are described in Section 9.4. Although degrons that are targeted by the Ub system are many and varied, their design is fundamentally similar to the multi-determinant organization of N-degrons, the first primary degradation signals in short-lived proteins to be discovered and analyzed^{4,5,9,17,19,24} (Fig. 11A).

The activity of N-degrons and other Ub-mediated degradation signals is a function of several variables^{5,9,17,19,168-171}. One of them is the efficacy of a degron's first determinant, i.e., a region of a protein substrate that is recognized by a cognate E3 Ub ligase. In the case of an N-degron that is targeted by the Arg/N-end rule pathway, this would be either an original or 'acquired' primary destabilizing (Nd^P) residue (Fig. 2A and Fig. 3). Residues downstream from a substrate's N-terminal Nd^P residue, and particularly a residue at position 2, can modulate the binding of an E3 N-recognin to an N-end rule substrate^{38,83,84}. If an initially formed N-degron (produced from a pre-N-degron) contains a secondary (Nd^S) or a tertiary (Nd^L) destabilizing residue, the efficacy of N-degron's first determinant would be determined, in addition, by the rate(s) of covalent modification(s) of the initial N-terminal residue that eventually yields an Nd^P residue that can be bound by a cognate N-recognin. Thus the levels and activity of Nt-amidasases and/or R-transferase (Fig. 2A and Fig. 3) would be expected to regulate the corresponding Nd^S/Nd^L-based N-degrons. Yet another parameter that influences these preliminary stages of N-degron's targeting is the extent of steric exposure of an Nd^P residue and the extent of flexibility of a protein's N-terminal region that would be expected to facilitate the binding of an Nd^P residue by an N-recognin^{5,9}.

Once the N-terminal Nd^P residue of a substrate is bound by a complex of N-recognin E3 and its associated Ub-conjugating (E2) enzyme, a race against time begins, given the transiency of the bound state and the necessity to produce a substrate-linked poly-Ub chain that is required for

downstream targeting steps. The synthesis of a poly-Ub chain (usually but not always a Lys⁴⁸-type chain) is initiated, in most cases, at an internal Lys residue of the substrate^{9,11,168}. In eukaryotes, this internal lysine is the second determinant of an N-degron (Fig. 11A). In some N-end rule substrates, only one internal lysine may be appropriately positioned for a kinetically efficacious attack on the thioester bond (denoted as ~) between E2 and Ub in the substrate-associated E3/E2~Ub complex. In other cases, including engineered N-end rule substrates^{9,20,24,168}, more than one Lys residue of a substrate is capable of such an attack. Its successful completion usually preempts participation by alternative lysines and is followed by a processive synthesis of a substrate-linked poly-Ub by a substrate-bound N-recognin E2/E3 Ub ligase. For example, there are two efficaciously 'ubiquitylatable' Lys residues, at positions 15 and 17, in an unstructured ~40-residue N-terminal extension of the 21-kDa mouse dihydrofolate reductase (DHFR), an engineered N-end rule substrate⁹. Either one of those lysines could be polyubiquitylated during the targeting, i.e., just one of two lysines (either one) had to be present for the activity of N-degron⁹. The otherwise identical but lysine-lacking N-terminal extension (with both lysines converted to Arg) did not result in the reporter's degradation by the Arg/N-end rule pathway, despite the presence of a destabilizing N-terminal residue⁹ (Fig. 11A).

In sum, the efficacy of a second-determinant Lys residue of a substrate's N-degron is determined by the lysine's spatial proximity to E2~Ub thioester of the targeting complex, and also by the extent of flexibility of a region containing the requisite lysine or lysines. This understanding of the necessity of an unstructured region (a requirement that is relevant to other Ub-dependent degrons as well) was produced by Bachmair and myself⁹ in 1989, in conjunction with the discovery, by Chau and colleagues in our laboratory, of the Lys⁴⁸-type poly-Ub chains and their necessity for protein degradation¹¹. We suggested that the main function of a substrate-linked poly-Ub chain is its physical binding to a specific site of the proteasome. By decreasing the rate of substrate dissociation, the resulting retention, through a poly-Ub chain, of a targeted substrate at the 26S proteasome would increase the probability of substrate unfolding and degradation by other components of the proteasome^{9,11}. In 1989, specific 'downstream' degradation steps and their mediator, the 26S proteasome, were just beginning to be defined experimentally, by several laboratories. With the above understanding of targeting in place by that time, it remained to be determined whether the demonstrated requirement for an unstructured region in N-end rule substrates⁹ was important only in the context of that region's polyubiquitylation. In an alternative mechanism, the same region, or a

similar (unstructured) but separate region of a substrate would also be required for a proteasome-mediated step that would initiate the processive degradation of a substrate that had been captured through its poly-Ub chain.

As predicted in 1989 (refs. 9,11), the 19S regulatory particle (RP) of the 26S proteasome was eventually shown to contain specific subunits that bind to a poly-Ub chain (ref. 54 and refs. therein). In one of possible models, the interaction of the 26S proteasome with a substrate-linked poly-Ub chain and the resulting delay in dissociation of the substrate from the proteasome would allow the ATP-dependent unfoldases of RP to unfold a previously structured region of the substrate and to insert it into the proteolytic chamber, thereby initiating processive proteolysis. An unfolded region could be, for example, the C-terminus or the N-terminus of substrate or, non-alternatively, an internal region, in which case it would be a hairpin loop whose insertion into the chamber would initiate processive proteolysis. The hairpin-insertion model of the proteasome-substrate interaction was suggested by us in 1989, and more explicitly in 1996. Fig. 11E illustrates the original model^{5,9,11}, which was proposed before the structural understanding of RP, its poly-Ub-binding subunits and other aspects of the 26S proteasome.

In this mechanism, an unstructured region of a substrate that encompasses its polyubiquitylated lysine may perform a 'double' duty of being important not only for polyubiquitylation but also for downstream, proteasome-mediated steps. *A priori*, it was also possible that the 26S proteasome might not strictly require an unstructured region in a substrate that had been captured through its poly-Ub chain, because unfoldases of RP might be able to initiate, efficiently enough, the ATP-mediated unfolding of a substrate (held by its poly-Ub chain) through thermal fluctuations alone, even in the absence of unstructured regions. Recent studies of N-degrons and other Ub-dependent degrons by the Matouschek, Jentsch, Dantuma, Coffino and other laboratories showed that the Ub-mediated protein degradation by the 26S proteasome does require the presence of an unstructured 'initiation' region in a substrate that has been captured by the proteasome through its poly-Ub chain. It was also shown that although the location of such a region in a polyubiquitylated substrate is not determined rigidly by the location of a branch point containing poly-Ub, the unstructured region should reside at an optimal distance from the branch point^{19,54,169,171-176}.

Thus the degradation, by the Arg/N-end rule pathway, of a protein containing an N-degron requires (i) the first determinant of N-degron, i.e., an N-terminal Nd^p residue that can be recognized by a cognate E3 N-recogin (an Nd^p residue is exposed by a proteolytic cut either directly or after

modifications of initially exposed Nd^{S} or Nd^{L} residues); (ii) the second determinant of N-degron, i.e., a Lys residue(s), which functions as the site of formation of a poly-Ub chain and usually resides in an unstructured region of a substrate; and (iii) a sterically 'suitable' unstructured region (either the same region that encompasses the Ub attachment site or another region) that serves as the initiation site for the unfolding of a captured substrate by the 26S proteasome^{9,169}. The latter requirement defines the third determinant of an N-degron. Yet another mechanistic aspect of N-degrons involves a complex of the chaperone-like ATPase Cdc48 (p97) with specific accessory proteins. This complex binds to several components of the Ub system, interacts with polyubiquitylated proteins 'upstream' of the 26S proteasome, and facilitates protein degradation in ways that are incompletely understood¹⁷⁷⁻¹⁷⁹. Despite these and other complexities, an N-degron can be an efficacious and portable degradation signal, capable of conferring extremely short (1-2 min) *in vivo* half-lives on either newly formed or conformationally mature proteins^{4,9,21,24}.

It is likely (but remains to be verified) that the degradation of naturally unstructured (disordered) eukaryotic N-end rule substrates, while still requiring the targeting by a cognate N-recognin E3, may not require ubiquitylation. This possibility is made particularly likely by the previously demonstrated physical affinity of both the Ubr1 E3 (N-recognin) and its associated Ufd4 E3 (Fig. 2A) for specific subunits of the 26S proteasome^{44,180,181}. Another setting in which eukaryotic N-degrons may also act analogously to Ub-independent prokaryotic N-degrons is the previously analyzed Ubr1-mediated cotranslational degradation of N-end rule substrates as they are being made, i.e., directly at the ribosome¹³⁹, apparently under conditions where the polypeptide chain that is being destroyed is targeted as a peptidyl-tRNA. The extent and mechanisms of the *in vivo* degradation of nascent proteins (i.e., of specific peptidyl-tRNAs) and newly formed (just completed) proteins, in comparison to degradation of the same proteins significantly after their synthesis^{21,24,139,182-185} is an extensively investigated but insufficiently understood subject. Given this problem's spatiotemporal and technical complexity, definitive advances in such studies would be likely to require new methods.

The understanding of degradation of N-end rule substrates by the Ub-independent bacterial Leu/N-end rule pathway (Fig. 5) is summarized in Fig. 12. It describes a detailed model proposed by Román-Hernández *et al.*⁸² and based on studies by the Baker, Sauer, Bukau, Maurizi and other laboratories^{16,75,76,78,81,82}. Most propositions of this model are supported by specific evidence, including crystal structures of ClpS, its mutant derivatives, and complexes of ClpS with peptide

mimics of N-end rule substrates^{73-75,79,186-189}; crystal structures of ClpA and the ClpAP protease¹⁹⁰⁻¹⁹²; and single-molecule measurements of protein translocation and degradation by the ClpAP-like ClpXP protease^{193,194}. The targeting begins when the ClpS N-recognin binds to an Nd^P residue of an N-end rule substrate (with K_d of $\sim 1 \mu\text{M}$) and delivers the substrate to one of six ClpA subunits of the ClpAP protease. ClpAP consists of the ClpA₆ unfoldase and the ClpP₁₄ protease, in a complex that includes the axial pore in the ClpA₆ hexamer that leads to the proteolytic chamber of the ClpA₆-associated ClpP₁₄ (Fig. 12).

A substrate-bound ClpS monomer interacts with ClpA of the ClpA₆ hexamer ~ 10 -fold more tightly than free ClpS does⁸². This binding preference ‘solves’ the problem of competition between free and substrate-bound ClpS for ClpS-binding sites on ClpA₆. In addition, the interaction between ClpS and ClpA₆ exhibits negative cooperativity, i.e., only one molecule of ClpS binds to ClpA₆ with high affinity⁷⁸. The 12-kDa ClpS consists of the flexible N-terminal (Nt) extension and the folded core domain, which binds to an N-degron (the N-terminal Nd^P residue) of an N-end rule substrate (Fig. 6B-D). The substrate-bound ClpS interacts with ClpA at the N-domain of ClpA. This domain is connected to the rest of ClpA by a flexible linker region. A key feature of the model is a conversion of the initial ‘low-affinity’ ternary complex (Fig. 12A) to a ‘high-affinity’ delivery complex (Fig. 12B, C), in which a part of the previously free Nt-extension of ClpS becomes bound to the ClpA₆ axial pore (near its entrance), thereby initiating the transfer of the ClpS-bound N-end rule substrate (its N-degron) from ClpS to a region inside the ClpA₆ pore. Previous evidence (ref. 195 and refs. therein) suggests that this region of the ClpA₆ pore exhibits a ClpS-like (but significantly weaker) affinity for a bulky hydrophobic (Nd^P) N-terminal residue. In the resulting arrangement, a conformational change of ClpA, fueled by ATP hydrolysis, pulls on the pore-bound Nt-extension of ClpS, perturbs the conformation of the core domain of ClpS and thereby both weakens its binding to N-degron of a substrate and facilitates the transfer of substrate (its N-degron region) from ClpS to a site in the ClpA₆ pore (Fig. 12C, D). As is also the case with eukaryotic N-end rule substrates, ‘optimal’ substrates of the bacterial Leu/N-end rule pathway contain features that are additional to the presence of a bulky hydrophobic N-terminal residue. These features include an unstructured region near the N-terminal destabilizing residue, an under-representation of acidic residues in that region, and a stretch of 2-3 hydrophobic residues 6-12 residues from the substrate’s N-terminus^{72,76,77}.

One experimentally supported assumption of the model is the possibility of more than one polypeptide chain occupying the pore of a ClpA-type unfoldase at the same time^{196,197} (Fig. 12C).

Another aspect of the model⁸² is resistance of the folded core of the ClpA-bound ClpS to unfolding by the ATP-fueled pull on the Nt-extension of ClpS (Fig. 12C). This resistance, accompanied by a conformational perturbation of ClpS, prevents degradation of the Nt-extension and the rest of ClpS, and in addition facilitates the dissociation of ClpS from ClpA, thereby leaving the Nd^p residue (N-degron) of the substrate inside the pore and completing the targeting cycle (Fig. 12D). Motor proteins operate through power strokes, through a biased Brownian motion (Brownian ratchet), or through a combination of these mechanisms^{193,198-200}. Experimental evidence favors a mechanism of the ClpA₆ motor that relies predominantly on a power stroke^{193,194}. Once the N-terminal region of an N-end rule substrate is brought inside the pore of the ClpA₆ motor (Fig. 12C, D), the ATP-dependent, presumably cyclic conformational changes of the pore region lead to a stepwise propulsion of the polypeptide chain of a captured substrate (accompanied by its unfolding) into the proteolytic chamber of the ClpA₆-associated ClpP₁₄. The result is processive degradation of the substrate to short peptides^{16,75,76,78,81,82}.

Ub-lacking proteolytic circuits such as, for example, the bacterial Leu-N-end rule pathway (Fig. 5, Fig. 6 and Fig. 12) are significantly less complicated, composition-wise and apparently also mechanism-wise, than Ub-dependent degrons and the targeting/degradation machinery in eukaryotes. The remarkable complexity (including compositional complexity) of the Ub system begs the question of how prokaryotes, which are obviously sophisticated about processive proteolysis^{200,201}, get by largely without Ub and ubiquitylation. Both bacteria and archaea have proteins that are spalogous (spatially similar¹) to Ub in that they contain the β -grasp Ub fold²⁰². Most archaea and some bacteria contain proteolytic pathways that involve conjugation of Ub-like proteins to other proteins^{66-70,203-207}. However, a large fraction of processive proteolysis in extant bacteria does not appear to be mediated by Ub-like pathways. For example, in the bacterial Leu/N-end rule pathway the ClpS N-recognin binds to N-end rule substrates and delivers them to the ClpAP protease for processive degradation without involving a ubiquitylation-like mechanism (Fig. 5 and Fig. 12).

One possibility is that the mechanistic complexity of the eukaryotic Ub system stems, at least in part, from its additional capabilities that are required in eukaryotes but not in prokaryotes. For example, some features of the subunit-selective proteolysis by the Ub system that are physiologically important in eukaryotes (Section 9.4) might not be necessary in bacteria^{13,168,208}. This conjecture remains to be verified. In addition, the ER-associated degradation (ERAD) of proteins that travel through or reside in the secretory pathway is functionally essential and Ub-mediated in

eukaryotes^{52,59,63,209} but a counterpart of this process in bacteria is Ub-independent. Specifically, bacteria employ sophisticated protein quality-control pathways in the periplasmic space (analogous to the ER lumen in eukaryotes) but do not appear to use ubiquitylation-like mechanisms.

It is also possible that an aspect of folding of some eukaryotic proteins (versus the presumed absence of such proteins in bacteria) presents a sufficient challenge to the eukaryotic 26S proteasome to require devices such as poly-Ub chains and associated machinery for destruction of such proteins. In other words, the Ub/proteasome-mediated processive proteolysis may have a greater protein-unfolding 'power' than analogous mechanisms that lack polyubiquitylation. This conjecture is not precluded but is unlikely to be a sufficient explanation, in part because the degradation of one and the same N-end rule substrate can be shown to be Ub-dependent in eukaryotes but Ub-independent in bacteria¹⁴. Specifically, Arg-e^K-βgalactosidase (Arg-e^K-βgal) is an engineered ~110-kDa (as a monomer) N-end rule substrate derived from *E. coli* βgal (LacZ) and produced, using the Ub fusion technique (Fig. 1B), through the cotranslational *in vivo* deubiquitylation of Ub-Arg-e^K-βgal^{4,9,21,24}. Arg-e^K-βgal bears N-terminal Arg, an Nd^P residue in eukaryotes and an Nd^S residue in bacteria (Fig. 2A and Fig. 4). Arg-e^K-βgal also contains, between N-terminal Arg and the βgal moiety, a ~40-residue region termed e^K [extension (e) containing lysine (K)]. The apparently unstructured e^K extension^{9,21,24} has the technically valuable property of lacking internal degrons while containing two 'ubiquitylatable' Lys residues, Lys¹⁵ and Lys¹⁷. Arg-e^K-βgal is polyubiquitylated and short-lived in *S. cerevisiae*, provided that its e^K extension contains at least one of these two lysines. Specifically, Arg-e^{AK}-βgal, in which both Lys¹⁵ and Lys¹⁷ were converted to (non-ubiquitylatable) Arg residues, is long-lived in *S. cerevisiae*, despite the presence of the N-terminal Arg residue^{9,24}. In contrast, both Arg-e^K-βgal and Arg-e^{AK}-βgal are degraded at similar rates by the *E. coli* Leu/N-end rule pathway¹⁴. Thus at least in this case, the ubiquitylation/proteasome machinery of eukaryotes 'imposes' the requirement of ubiquitylation that is unnecessary in bacteria, vis-à-vis the same reporter protein.

Yet another, non-alternative possibility is that the Ub system might have indeed been 'overdesigned' in the course of eukaryotic evolution, i.e., that bacterial-type mechanisms, with fewer components and without extensive use of ubiquitylation, can accomplish what the Ub system does. An overdesign might have happened for reasons, suggested by Lynch²¹⁰, that are generally relevant to evolution of eukaryotes. A population genetics-based argument can be made that many aspects of molecular circuits in eukaryotic cells have evolved, at least initially, through a quasi-random,

recurrent genetic drift. The relative importance of evolution by this route (as distinguished from adaptive Darwinian evolution through positive selection) depends on the breeding system of a species, on the organization of its genome, and on its history of long-term population bottlenecks. A small population size is likely to have been a recurrent characteristic of early eukaryotic evolution, at the time of emergence of a 'stem' eukaryote, roughly 1.5 billion years ago. Most new alleles of a genetic locus are deleterious. The probability of fixation of a new allele (vis-à-vis the probability of its disappearance from a population) is higher in a smaller population²¹⁰. Under such conditions, complicated circuits might have evolved, to a significant extent, through occasional fixations of mildly deleterious mutations that were eventually 'compensated' by suppressor mutations that often had their own fitness costs and were compensated by yet additional suppressors.

In sum, complexity in designs of biological circuits is not always a sign of adaptive evolution alone, because complexity can also result from a recurrent, long-term, sometimes deleterious genetic drift. (The relatively large sizes and numbers of introns in mammals, vis-à-vis much lower sizes and numbers of introns in fungi and their absence in prokaryotes are one example of evolution, in multicellular eukaryotes, that is partially drift-based and population size-dependent²¹⁰.) The history of a species contains, in differing proportions, both a drift mode and an adaptive (Darwinian) mode. The population sizes of prokaryotes are (and were) vastly larger than those of eukaryotes, indicating that populations of prokaryotes are much more resistant than eukaryotes to evolutionary changes that stem from genetic drift, as distinguished from positive selection²¹⁰. If the primordial Ub system emerged initially to a large extent through a genetic drift, this would account, at least in part, for the absence of the *bona fide* Ub system in extant prokaryotes. The *S. cerevisiae* and human Ub systems are remarkably similar, differing mostly by a larger number of human E3 Ub ligases and associated machinery. Thus the design of the Ub system must have reached its essentially modern state in early eukaryotes, before the separation of fungal and animal lineages. In sum, although an adaptive Darwinian evolution undoubtedly played a major role in making the extant Ub pathways what they are, it is likely that the Ub system emerged and became 'entrenched' in primordial eukaryotes to a large extent through a genetic drift unaccompanied (at the beginning) by a significant positive selection⁸⁰, a general pattern of evolution discussed by Lynch²¹⁰.

Because an N-degron consists of several determinants, subunit-selective polyubiquitylation and degradation of an oligomeric protein can occur not only in *cis* (with all determinants of an N-degron in the same polypeptide chain) but also in *trans*¹³ (Fig. 11B, C). In the resulting

subunit-selective protein degradation, discovered in 1990 in the context of the Arg/N-end rule pathway and found to be characteristic of the entire Ub system as well, a destabilizing N-terminal residue of one protein subunit can be engaged by an N-recognin to direct selective degradation of another subunit in the same oligomeric protein¹³. This capability makes possible protein remodeling, a major functional attribute of the Ub system. Regulatory circuits wield subunit-selective proteolysis for either positive or negative control, including transitions in the cell cycle, the control of transcription and DNA replication, and many other processes. Among specific examples are the activation of a major transcription factor NF- κ B, through the degradation of its inhibitory subunit I κ B, and the inactivation/alteration of cyclin-dependent kinases (which drive the cell cycle) through the subunit-selective degradation of their cyclin subunits^{211,212}. Matouschek and colleagues have shown that the location and properties of an unstructured region of a protein substrate that serves as the proteasome-initiation site can determine which subunit is degraded^{19,168}. For example, a polyubiquitylated subunit of an oligomeric protein that is delivered to the 26S proteasome via its poly-Ub chain can be resistant to degradation and direct the subunit-selective destruction of a non-ubiquitylated subunit in the same oligomeric protein¹⁶⁸. In the cited study, this *trans*-degradation effect¹⁶⁸ was demonstrated with substrates of the Ub-fusion degradation (UFD) pathway (see Section 9.1). Given the likely generality of this mechanism, Fig. 11D illustrates the (hypothetical) possibility of a *trans*-degradation of this kind for an oligomeric substrate of the N-end rule pathway.

Another important property of N-end rule circuits is exemplified by the *S. cerevisiae* Ubr1 E3 N-recognin. The 225 kDa Ubr1 (Fig. 7A) contains at least four substrate-binding sites. Two of them recognize specific N-degrons, whereas the other sites recognize internal (non-N-terminal) degrons. The occupancy of Ubr1 sites that recognize N-degrons has been shown to regulate, in physiologically relevant ways, the activity of another binding site of the Ubr1 N-recognin^{27,29,38-40} (Section 9.4). The multiplicity of substrate-binding sites in Ubr1 and their allosteric dependencies are likely to recur in other E3 N-recognins as well.

7. Applications of the ubiquitin fusion technique and N-degrons

The Arg/N-end rule pathway (Fig. 2A) was discovered through the invention of the Ub fusion technique^{4,9} (Fig. 1B). This strategy was later employed to identify the bacterial Leu/N-end rule pathway as well, via expression of the yeast Ubp1 deubiquitylase in *E. coli*¹⁴. The Ub fusion technique is still the method of choice for expressing, *in vivo*, a protein of interest that bears a desired

N-terminal residue^{20,24,213} (Fig. 1B). Over the last two decades, this approach gave rise to several ‘descendant’ technologies, in the Ub field and beyond^{6,139,213-219}. One example is a heat-activated, portable N-degron that allows construction of temperature-sensitive (*ts*) variants of specific proteins without changing their amino acid sequences^{216,220}. Another example is the Ub sandwich assay, in which a linear fusion of three reporter domains that bears N-terminal Ub as well as Ub moieties between the domains is designed to detect and measure cotranslational protein degradation in living cells¹³⁹. Yet another technique, the Ub translocation assay (UTA), employs Ub fusions that contain N-terminal signal sequences to probe kinetic aspects of protein translocation across membranes *in vivo*²¹⁷. In 1994, Johnsson and I developed a method, later termed the protein fragment complementation assay (PCA), that detects protein interactions through the conditional *in vivo* reconstitution of a single-domain protein from its fragments²¹⁸. The first *in vivo* PCA involved a split-Ub design in which two halves of a mutant Ub moiety were configured as sensors of interactions between two proteins of interest that were linked to Ub halves^{218,221}. The split-Ub PCA led to other useful PCAs by other laboratories, including split GFP (green fluorescent protein), split DHFR, and split β -lactamase (refs. 222-225 and refs. therein).

The Ub fusion technique also led to the concept of signal regulated, cleavage-mediated toxins (sitoxins), in which an initially short-lived (or otherwise down-regulated) toxin is activated through a cleavage (e.g., by a viral protease in a virus-infected cell) that removes a degron or activates a toxin otherwise, thereby increasing the level of active toxin²²⁶. Variants of this strategy were employed by others to develop protein-based conditional reagents, including antiviral drugs²²⁷⁻²³³. Muir and colleagues combined a version of the sitoxin approach²²⁶ with the split-Ub PCA method²¹⁸ to develop the SURF (split Ub for the rescue of function) technique, in which a protein of interest that is made short-lived *in vivo* through a link to a portable degron can be metabolically stabilized by a small compound that causes a cleavage-mediated removal of degron²³⁴. Using mouse DHFR as a reporter, we showed that the Ub-dependent proteolysis of a degron-bearing protein can be slowed down or halted through the addition of a compound (e.g., methotrexate, a tight-binding DHFR inhibitor) that increases thermodynamic stability of a protein^{235,236}. Wandless and colleagues employed this approach, using *E. coli* DHFR and its inhibitor trimethoprim, to develop a method for regulating protein degradation in cultured mammalian cells and intact animals, including their neurons²³⁷. Another recent technique uses a conditionally expressed viral TEV protease to cleave, *in vivo*, a protein of interest that had been engineered to contain a TEV cleavage site at a desired position²³⁸.

This method makes it possible, among other things, to create an N-degron posttranslationally, as distinguished from the cotranslational production of N-degrons through the Ub fusion technique^{4,9,213}.

8. The Ac/N-end rule pathway

The Ac/N-end rule pathway³ (Fig. 2B and Fig. 4A) was discovered 24 years after the Arg/N-end rule pathway⁴ (Fig. 2A). In *S. cerevisiae*, ^{Ac}N-degrons of the Ac/N-end rule pathway are recognized by the Doa10 E3 Ub ligase³ and apparently by other E3s as well (C.-S. Hwang, A. Shemorry and A.V., unpubl. data). The 151-kDa RING-type Doa10 E3 is a multispinning integral membrane protein located in the endoplasmic reticulum (ER) membrane and in the inner nuclear membrane²³⁹⁻²⁴¹. The Doa10 E3 functions together with the Ubc6 and Ubc7 E2 enzymes, and targets both 'soluble' (nuclear and cytosolic) and transmembrane proteins^{3,242}. Isolated *S. cerevisiae* Doa10 selectively binds to the Nt-acetylated N-terminal Met, Ala, Val, Ser, Thr, Cys, Gly and Pro residues of model peptides³. Remarkably, the binding of Doa10 to Nt-acetylated residues of these peptides is precluded by the presence of a basic residue such as Lys at position 2 (ref. 3). Thus the *S. cerevisiae* Ac/N-end rule pathway avoids the targeting of proteins with a basic residue at position 2 through two independent constraints: first, such proteins are not Nt-acetylated in yeast^{91,92}; and second, the cognate Doa10 E3 apparently does not recognize Nt-acetylated proteins that bear a second-position basic residue³. Evolutionary and mechanistic reasons for a 'double' filter against potential substrates of this class are unclear at present, because the absence of Nt-acetylation of such proteins in *S. cerevisiae* should suffice. Mammalian E3s that are sequelous (similar in sequence¹) to *S. cerevisiae* Doa10 include the human TEB4 E3 Ub ligase²⁴³. This and other evidence (C.-S. Hwang, B. Wadas and A.V., unpubl. data) indicate the presence of the Ac/N-end rule pathway in multicellular eukaryotes as well.

Nt-acetylation is largely cotranslational, apparently irreversible, and involves a majority of cellular proteins. What functions are subserved by such a massive production of degradation signals (^{Ac}N-degrons) in nascent proteins, if many of these proteins are destined for long half-lives? We suggested that a major role of these degradation signals involves quality-control mechanisms and the regulation of protein stoichiometries in a cell³. A key feature of such mechanisms would be conditionality of ^{Ac}N-degrons. If a nascent Nt-acetylated protein can fold its N-terminal domain rapidly enough, or if this protein interacts with a 'protective' chaperone such as Hsp90, or becomes assembled into a cognate multisubunit complex, the cotranslationally created ^{Ac}N-degron of this

protein may become inaccessible to a cognate Ub ligase such as Doa10. Consequently, this Nt-acetylated protein would not be targeted for degradation via its ^{Ac}N-degron. In contrast, delayed or defective folding of a protein's N-terminal domain (because of oxidative, heat or other stresses; or a conformation-perturbing mutation; or nonstoichiometric levels of cognate protein ligands) would keep an ^{Ac}N-degron exposed (active) and thereby would increase the probability of the protein's destruction.

Might the conditionality of ^{Ac}N-degrons underlie the regulation of protein assembly into multiprotein complexes? For example, histones and ribosomal proteins tend to be short-lived until they become integrated into larger assemblies, the nucleosomes (in chromosomes) and the ribosomes, respectively^{244,245}. Selective degradation of these proteins in their unassembled states makes possible the regulation of their levels vis-à-vis the rates of their production through transcription and translation. Little is known about degrons of ribosomal proteins or histones that mediate their conditional degradation. At the same time, most histones and ribosomal proteins are Nt-acetylated^{91,92}. We suggested that the regulation of *in vivo* protein stoichiometries in oligomeric proteins and nucleoproteins (including chromosomes and ribosomes) through the assembly-controlled protein degradation may be mediated, to a large extent, by ^{Ac}N-degrons that form cotranslationally and are accessible to a cognate Ub ligase in 'free' proteins but not in their 'assembled' counterparts³.

Aneuploidy, in which the chromosome number in a cell is not an exact multiple of the haploid number, is a frequent property of cancer cells and a cause of birth defects such as Down syndrome (a trisomy of chromosome 21). Physiological defects in aneuploid cells are likely to be caused, at least in part, by maladaptive molar ratios of newly formed proteins in such cells, given their deviations from wild-type gene dosages on over-represented or under-represented chromosomes. Homeostatic responses in such cells would have to destroy a higher than normal load of unassembled proteins. This may account for the known hypersensitivity of aneuploid cells to proteasome inhibitors²⁴⁶. If so, and if ^{Ac}N-degrons contribute to proteolysis that regulates *in vivo* protein stoichiometries, aneuploid cancer cells may prove to be hypersensitive to inhibition of the Ac/N-end rule pathway, a mode of therapy that would be more selective than proteasome inhibitors, which down-regulate the Ub system at large.

Nonlinear effects of *in vivo* protein degradation are a rule rather than exception. For example, if a homodimeric protein is significantly longer-lived *in vivo* in comparison to its monomer subunit (because a degron accessible in a monomer becomes buried or otherwise less active in a dimer), the

steady-state concentration of this protein in a cell can be a strongly nonlinear (sigmoid) function of the rate of its synthesis. Such effects would occur with both homooligomers and heterooligomers. In what follows, I mention a potentially important aspect of this disposition that has not been pointed out previously, to my knowledge. Specifically, one should expect, in aneuploid and other 'disbalanced' settings, a major difference in physiological outcomes for homooligomers versus heterooligomers. Consider, for example, an aneuploid cell that overproduces at least some proteins from its over-represented chromosome. If a specific overproduced protein forms a heterodimer with a protein encoded by another chromosome that is present at the normal dosage, an excess of overproduced protein would have its degron, e.g., an ^{Ac}N -degron, exposed (active) and would be preferentially destroyed by the Ac/N-end rule pathway. As a result, the *in vivo* concentration of this overexpressed protein would be at most marginally higher than it would have been in the absence of aneuploidy. By contrast, if an overproduced protein forms a homodimer (or a homooligomer) in which an ^{Ac}N -degron is inactive (sterically shielded), the Ac/N-end rule pathway would be unable to 'detect' an overexpression of this (self-associated) protein, with the consequent failure of proteolysis-based compensatory mechanisms. The same argument applies to degradation signals distinct from ^{Ac}N -degrons, as long as a degron is rendered inactive (or less active) through the formation of a homooligomeric protein. This argument can also apply if a heterodimer is formed from subunits encoded by the same over-represented chromosome. In sum, it is possible (this conjecture remains to be verified) that fitness-reducing effects of aneuploidy stem, to a significant degree, not only from a disbalanced (non-stoichiometric) production of proteins, but also specifically from the impossibility, for an otherwise protective proteolytic circuit, to 'detect' an overproduction of proteins that form homooligomers.

The irreversibility of Nt-acetylation may underlie a putative role of ^{Ac}N -degrons in the degradation of relatively long-lived intracellular proteins, with half-lives of many hours or days. The initial burst of degradation, through an ^{Ac}N -degron, of some among the molecules of a newly formed, not yet 'assembled' Nt-acetylated protein is followed by the formation of a cognate oligomeric complex in which the protein's ^{Ac}N -degron becomes inactive (sterically shielded). Whether or not this ^{Ac}N -degron is activated (becomes accessible) later would be determined by the probability of dissociation of the complex over time, either a spontaneous dissociation or an 'induced' one. The latter can be caused, for example, by a physiologically relevant phosphorylation of the complex, or by events such as heat or oxidative stress. The resulting transient accessibility of the (now exposed)

^{Ac}N-degron of a protein would immediately and strongly increase the probability of the protein's destruction, until the cognate complex can reassemble and thereby again protect the protein. In contrast to the notion of a low and time-invariant probability of destruction of a long-lived protein, the above model posits recurrent transitions between at least two states. In one state, the probability of protein's destruction is high (^{Ac}N-degron is exposed). In the other state, in which the protein is a part of a complex, the probability of protein's destruction is low or negligible. The kinetics of reversible transitions between these states and the probability of protein's destruction in the (transient) absence of protective complex would determine, together, an experimentally measured *in vivo* decay curve of such a protein. Thus an apparently first-order degradation kinetics may be result, mechanistically, from transitions between different structural states of a protein and different probabilities of degradation. This model, which remains to be verified for ^{Ac}N-degrons, is relevant to other degrons as well.

In the recently determined crystal structure, by the Barford laboratory, of a complex between the Hcn1 and Cut9 subunits of the yeast *Schizosaccharomyces pombe* APC/C Ub ligase, the Nt-acetylated Met residue of Hcn1 was found to be enclosed within a chamber created by the Cut9 subunit²⁴⁷ (Fig. 13). The authors interpreted this result as a likely example of the assembly-mediated shielding of ^{Ac}N-degrons³. Although Protein Data Bank (PDB) and other databases of protein structures contain thousands of proteins, the Hcn1-Cut9 complex²⁴⁷ is one of very few (< 10) structures that show details of a protein-bound Nt-acetylated residue. Another structure of a complex with a known spatial location of Nt-acetylated N-terminus contains the tandem PHD1/2 finger (a part of the human DPF3b chromosomal protein) that is bound to the Nt-acetylated peptide of histone H4 (residues 1-22) and specifically interacts with several residues of this H4 peptide, including its Nt-acetylated Ser¹ residue²⁴⁸. The paucity of such structures in current databases stems from often unstructured N-terminal regions of studied proteins, from frequent N-terminal truncations of proteins (to facilitate their crystallization), and from the still prevalent use, for crystallization, of eukaryotic proteins expressed in bacteria, in which Nt-acetylation is not efficacious. Hcn1-Cut9 and analogous complexes in which the shielding of specific Nt-acetylated N-termini can be demonstrated at a near-atomic resolution²⁴⁷, can now be used to explore physiological functions of ^{Ac}N-degrons.

For reasons described above, ^{Ac}N-degrons comprise the most prevalent class of specific degradation signals in cellular proteins³ (Fig. 2B and Fig. 4). Although the Nt-acetylation of cellular proteins by ribosome-associated Nt-acetylases is largely cotranslational^{89,95}, the (currently unknown)

molar concentrations of specific Nt-acetylases *in vivo* are likely to be significantly lower than the molar concentration of ribosomes. This, and the inherent stochasticity of cotranslational enzymatic reactions make it virtually certain that Nt-acetylation of proteins is less than quantitative in most cases. Indeed, many proteins are known to be incompletely Nt-acetylated^{91,110}, despite the existence of posttranslational Nt-acetylation (J.-H. Oh and A.V., unpubl. data). The latter may not be able to compensate for the incompleteness of cotranslational Nt-acetylation because of rapid formation of cognate complexes in which the N-termini of proteins, either Nt-acetylated (Fig. 13) or unacetylated, would become sterically inaccessible. Given this and other aspects of Nt-acetylation, the emerging field of the Ac/N-end rule pathway may prove to be a source of new answers to several outstanding questions. For example, the phenomenon of proteotoxicity might stem, in part, from the incomplete Nt-acetylation of normally expressed proteins^{91-93,102} and the resulting presence, in a cell, of otherwise identical protein molecules that either lack or contain an ^{Ac}N-degron. Given this possibility³ and putative functions of ^{Ac}N-degrons, the Ac/N-end rule pathway may also play a role in processes that alter the rate of cellular and organismal aging.

9. The Arg/N-end rule pathway

The Arg/N-end rule pathway involves the Nt-arginylation of N-end rule substrates and also the targeting of specific unmodified N-terminal residues by UBR-type E3 N-recognins, which can recognize not only N-degrons but also internal (non-N-terminal) degrons (Fig. 2A and Fig. 3). Despite their broad range, the known functions of the Arg/N-end rule pathway (Section 3) are still the tip of the iceberg. Some of these functions are understood, in part, both physiologically and mechanistically (Section 9.4), but many roles of the Arg/N-end rule pathway were discovered through genetic approaches and therefore remain to be explicated in terms of specific substrates and mechanisms. The known physiological substrates of the Arg/N-end rule pathway are the beginning of a much longer list. It already contains a number of putative (unverified) substrates and may require new methods for its systematic elucidation (Section 5 and Fig. 10).

9.1. The double-E3 design of the Arg/N-end rule pathway

Until recently, polyubiquitylation of substrates by the *S. cerevisiae* Arg/N-end rule pathway was thought to be mediated by a dimer comprising the 225-kDa Ubr1 E3 and the Ubr1-bound 20-kDa Rad6 E2 enzyme^{5,6,38,39,249}. In 2010, it was discovered that the targeting ensemble is more elaborate. It comprises a physical complex of the RING-type Ubr1 E3 (N-recognin) and the HECT-type Ufd4

E3, in association with their cognate E2s Rad6 and Ubc4/Ubc5, respectively^{40,44} (Fig. 2A). Ufd4 is the 168 kDa HECT-type E3 of the Ub-fusion degradation (UFD) pathway that recognizes a 'nonremovable' N-terminal Ub moiety of a Ub fusion as a primary degron and polyubiquitylates the Ub moiety, a prerequisite for the fusion's degradation by the 26S proteasome^{180,181,250-255}. The UFD pathway was discovered through analyses of N-terminal Ub fusions in which an alteration of either the Ub moiety or a junctional amino acid residue inhibits the cleavage of a fusion by deubiquitylases and thereby results in the fusion's degradation by the UFD pathway^{4,250,256,257}. The UFD pathway is present in both yeast and mammals²⁵⁸, suggesting that the Ubr1-Ufd4 double-E3 organization of the *S. cerevisiae* Arg/N-end rule pathway is universal among eukaryotes⁴⁴.

Ufd4 is not an N-recognin, i.e., it does not, by itself, recognize N-degrons, in contrast to Ubr1. However, through its physical interaction with the Ubr1 E3 (Fig. 2A), the Ufd4 E3 functions as a novel component of the Arg/N-end rule pathway that increases the efficacy of Ubr1, at least in part by augmenting the processivity of polyubiquitylation of N-end rule substrates⁴⁴. The function of Ufd4 in the Arg/N-end rule pathway is not confined to processivity enhancement, as Ufd4 can also recognize the internal degron of the Mgt1 DNA repair protein (a substrate of the Arg/N-end rule pathway) even in *ubr1*Δ cells⁴⁰, i.e., in the absence of Ubr1. Thus the sets of internal degrons recognized by Ubr1 and Ufd4 are partially overlapping. Although Ufd4 is not strictly essential for the ability of Ubr1 to mediate the Arg/N-end rule pathway, this pathway is detectably impaired in *ufd4*Δ cells⁴⁴.

Earlier studies introduced the operationally defined concept of an E4 as an E3-like enzyme that cooperates with substrate-specific ubiquitylation machinery to increase the efficacy (including processivity) of polyubiquitylation, and in some cases alters topology of a growing poly-Ub chain, i.e., the locations of Gly-Lys isopeptide bonds between the adjacent Ub moieties in a chain^{257,259-261}. Remarkably, the increased processivity of the Ubr1-dependent polyubiquitylation of N-end rule substrates by the Ubr1-bound Ufd4 Ub ligase⁴⁴ is 'reciprocated' in the context of the UFD pathway. Specifically, the Ufd4-bound Ubr1 Ub ligase increases the processivity of polyubiquitylation of UFD substrates⁴⁴. Thus, operationally, the complex of Ubr1 and Ufd4 (Fig. 2A) functions as an E3-E4 pair in which the 'assignment' of an E3 or E4 function depends on a substrate and the nature of its degron. It was also found that Ubr1, similarly to Ufd4, contains a domain that specifically binds to the N-terminal Ub moiety of protein fusions but not to free Ub, indicating that Ubr1 can target UFD substrates independently of the Ufd4 Ub ligase⁴⁴. Because Ubr1 is apparently ~10-fold less abundant

than Ufd4 in wild-type yeast⁴⁴, the ‘double’ E2-E3 Ubr1/Rad6-Ufd4/Ubc4 complex is expected to mediate the bulk of the Arg/N-end rule pathway (Fig. 2A), whereas the same complex mediates only a part of the UFD pathway. *In vitro*, Ubr1 and Ufd4 bind, separately and independently, to specific (partially overlapping) sets of subunits of the 26S proteasome¹⁸⁰. Now that the proteasome-binding *in vivo* entity turned out to be, most likely, the Ubr1-Ufd4 complex (with associated E2 enzymes), it is possible that the targeting of N-end rule and UFD substrates is mediated, at least in part, by this complex that is ‘pre-bound’ to the 26S proteasome. These are just some of the ramifications of the discovery of the Ubr1-Ufd4 complex, a functional and mechanistic advance that unified two proteolytic systems that have been studied separately for two decades⁴⁴.

Purified *S. cerevisiae* Ubr1 binds to either type-1 (basic) or type-2 (bulky hydrophobic) destabilizing N-terminal residues of 11-residue peptides with a K_d of $\sim 1 \mu\text{M}$, whereas no binding ($K_d > 0.1 \text{ mM}$) was observed with otherwise identical peptides bearing the N-terminal Gly residue^{38,43}. Crystal structures of the ~ 80 -residue UBR domains (type-1 binding sites) of *S. cerevisiae* Ubr1 and its mammalian counterpart have been determined (Fig. 8A, B)⁸³⁻⁸⁵, but no structural information is available about the type-2 binding site, let alone the entire ~ 200 -kDa mammalian or yeast Ubr1, despite crystallization attempts by more than one laboratory. The discovery of interaction between the Ubr1 and Ufd4 E3s suggests that their complex⁴⁴ might be easier to crystallize than its individual components. Although the type-1 and type-2 binding sites are adjacent in *S. cerevisiae* Ubr1 (Fig. 7A), a screen for mutations in *UBR1* that inactivate one but not the other binding site has readily yielded Ubr1 mutants with such properties³⁸, suggesting a structurally autonomous folding not only of the type-1 (UBR) domain (Fig. 8A, B) but of a type-2 domain as well.

A spatial proximity of the type-1 and type-2 binding sites of Ubr1 was made use of in designing a bivalent inhibitor of the N-end rule pathway that was expected to bind simultaneously to both of these sites and therefore to exhibit a higher efficacy than a ‘monovalent’ inhibitor. In a proof-of-principle study, a tetramer of X- β gal, produced from Ub-X- β gal (Fig. 1B; X=Arg or Leu), was produced as a set of homotetramers and heterotetramers by coexpressing, in *S. cerevisiae*, Arg- β gal (a type-1 N-end rule substrate) together with Leu- β gal (a type-2 N-end rule substrate)²⁶². These engineered proteins contained a ~ 40 -residue extension between N-terminal residue and the β gal moiety. This sequence, termed the e^{AK} extension (see Section 6), lacked Lys residues, and the first 200 residues of the β gal moiety also lacked lysines. As a result, these β gal-based reporters were long-lived *in vivo*, despite their destabilizing N-terminal residues^{9,24,213}. In the 3D structure of *E. coli*

β gal, two of its N-termini (with the other pair of N-termini on the opposite side of the tetramer) are spatially close and oriented in the same direction²⁶³. This spatial feature of β gal and the above design resulted in some of expressed X- β gal tetramers being heterotetramers in which two flexible, adjacent N-terminal regions contained the N-terminal Arg and Leu residues²⁶². It was found that these 'bivalent' Arg/Leu- β gal heterotetramers inhibited the degradation of reporter N-end rule substrates in *S. cerevisiae* much more efficaciously than either Arg- β gal alone or Leu- β gal alone²⁶². An inhibitor of this kind acts as a competitive inhibitor of the type-1/2 sites of N-recognins and may also act, at the same time, as an allosteric *activator* of other substrate-binding sites of N-recognins, the ones that bind to internal (non-N-terminal) degrons of specific regulatory proteins. The latter inference (it remains to be verified for macromolecular-size inhibitors) stems from the known allosteric activation, by dipeptides with destabilizing N-terminal residues, of the autoinhibited third substrate-binding site of yeast Ubr1, the site that targets the internal degon of the Cup9 transcriptional repressor (Fig. 15 and Section 9.4). The Kwon laboratory has further advanced the bivalent-inhibitor approach by producing and characterizing small (< 1 kDa) synthetic heterovalent inhibitors of the N-end rule pathway²⁶⁴.

In contrast to *S. cerevisiae*, in which Ubr1 is the sole N-recognin of the Arg/N-end rule pathway, the genome of a multicellular eukaryote, e.g., a mammal, encodes at least four UBR domain-containing E3 N-recognins, termed Ubr1, Ubr2, Ubr4, and Ubr5 (also called Edd)^{15,31,43} (Fig. 8C). Sequelogs¹ of the 570-kDa mouse Ubr4 N-recognin are known as PUSHOVER in *D. melanogaster* and as BIG in plants^{15,31,265-269}. Mammalian Ubr1 and Ubr2 are highly sequelogous N-recognins (they are also sequelogous to *S. cerevisiae* Ubr1), whereas the sequelogy between, e.g., Ubr1 and either Ubr4 or Ubr5/Edd is largely confined to their ~80-residue UBR domains. Mutational inactivation of human UBR1 causes Johanson-Blizzard syndrome (JBS) (Sections 3 and 9.4), and *Ubr1*^{-/-} mice exhibit aspects of JBS as well³⁴. Mice lacking Ubr2 have a variety of defects, including genomic instability and impaired spermatogenesis (and infertility) in males^{30,123}. One function of mouse Ubr2 in meiotic spermatocytes is ubiquitylation of histone H2A, in a setting where this modification plays a role in transcriptional silencing²⁷⁰. In contrast to viability of single-mutant *Ubr1*^{-/-} and *Ubr2*^{-/-} mice, double-mutant mouse strains that lacked both Ubr1 and Ubr2 died as ~11-day (midgestation) embryos, with major defects in neurogenesis and cardiovascular development²⁷¹.

Mammalian Ubr1, Ubr2 and Ubr4 N-recognins contain both type-1 and type-2 substrate-binding sites, whereas the Ubr5/Edd N-recognin lacks the type-2 binding site³¹ (Fig. 8C). Thus, in agreement with findings by the Bachmair laboratory about plant N-recognins^{18,272,273}, a mammalian N-recognin can contain both type-1 and type-2 substrate-binding sites or just one of them. The presence of a UBR domain in an E3 Ub ligase signifies its N-recognin property (i.e., its ability to bind to N-terminal Nd^p residues of proteins or short peptides; Fig. 3) only in some cases, as the mouse Ubr3, Ubr6 and Ubr7 E3s are not N-recognins, despite the presence of a UBR domain^{43,274} (Fig. 7 and Fig. 8). The *S. cerevisiae* Ubr2 E3, which contains a UBR domain, is also not an N-recognin^{43,275}. Cognate *in vivo* ligands of UBR domains of non-N-recognin E3s remain to be identified. The identical names of mammalian Ubr2 and *S. cerevisiae* Ubr2 are functionally incongruent, in that only mammalian Ubr2 is an N-recognin.

Studies with mice, flies and plants indicated a broad range of physiological functions of Ubr4 and Ubr5/Edd (refs. 15,31,265-269 and refs. therein), but analyses of these E3s vis-à-vis their functions as N-recognins have yet to take place. Specific interactions of the type-1/2 binding sites of the *S. cerevisiae* Ubr1 N-recognin with dipeptides bearing Nd^p residues were shown to allosterically activate a third binding site of Ubr1, thereby accelerating the degradation of its target, Cup9, in a circuit that 'senses' the presence of extracellular peptides and accelerates their uptake^{27,29,138} (Fig. 15). It would be, therefore, particularly informative to determine whether the analogous N-degron-binding sites of, e.g., mammalian Ubr4 and Ubr5/Edd also function to regulate, allosterically, the activity of other substrate-binding sites of these E3s.

A recent advance²⁷⁶ opened up yet another dimension in studies of the mammalian N-end rule pathway. As in the rest of the Ub system, the 'upstream' steps of polyubiquitylation of mammalian N-end rule substrates are mediated by the Ub-activating (E1) enzyme Uba1, which transfers the activated Ub moiety to cognate E2 enzymes such as Ube2A/Ube2B (the mammalian counterparts of the *S. cerevisiae* Rad6 E2). It was shown that Uba6-Use1, a different pair of E1-E2 enzymes, also functions in the Arg/N-end rule pathway, in that the Use1 E2 of this pair is a component of 'alternative' E2-E3 Ub ligases that contain UBR-type N-recognins such as Ubr1 and Ubr2²⁷⁶.

9.2. N-terminal deamidation in the Arg/N-end rule pathway

In *S. cerevisiae*, the deamidation branch of the Arg/N-end rule pathway is mediated by the *NTA1*-encoded 52-kDa Nt^{N,Q}-amidase. Nta1 can deamidate either N-terminal Asn (N) or N-terminal Gln (Q), converting them into Asp or Glu, respectively, and thereby enabling the Nt-arginylation of

resulting proteins by the Ate1 R-transferase²³ (Fig. 2A). Although there is enough of Nta1 in the yeast cytosol (and possibly in the nucleus as well) to mediate the deamidation branch of the Arg/N-end rule pathway²³, the bulk of Nta1 was shown to reside in mitochondria (<http://yeastgfp.yeastgenome.org/>), in agreement with our findings (H.-R. Wang and A.V., unpubl. data). *NTA1* apparently encodes both forms of Nt^{N,Q}-amidase, as there are two alternative start (ATG) codons in the *NTA1* open reading frame (ORF), with a putative mitochondrial presequence between them²³. Physiological substrates of the *S. cerevisiae* Nta1 Nt^{N,Q}-amidase are unknown. Apart from the inability of *nta1Δ* yeast cells (in contrast to wild-type cells) to degrade engineered cytosolic N-end rule substrates that bear N-terminal Asn or Gln, no defects could be detected, thus far, with *nta1Δ* cells, including the apparent absence of defects in their mitochondria (ref. 23; H.-R. Wang and A.V., unpubl. data). One possibility is that the mitochondrial pool of Nta1 may function in the recently discovered mitochondrial N-end rule pathway^{277,278} (Section 11).

In contrast to *S. cerevisiae*, where a single Nt^{N,Q}-amidase deamidates both N-terminal Asn and N-terminal Gln (Fig. 2A), the deamidation branch of the Arg/N-end rule pathway is bifurcated in multicellular eukaryotes. Specifically, the mammalian *Ntan1*-encoded 35-kDa Nt^N-amidase deamidates solely N-terminal Asn, whereas the *Ntaq1*-encoded 24-kDa Nt^Q-amidase deamidates solely N-terminal Gln^{25,42,116,117} (Fig. 3). Mouse *Ntan1* and *Ntaq1* are present largely in the cytosol and the nucleus^{25,42}, in contrast to the largely mitochondrial location of *S. cerevisiae* Nta1. *Ntan1*^{-/-} mice, which lack Nt^N-amidase and cannot degrade N-end rule reporters bearing N-terminal Asn, are hypokinetic and have a significantly impaired spatial memory²⁵. The recently constructed *Ntaq1*^{-/-} mouse strains, which lack Nt^Q-amidase and cannot degrade N-end rule reporters bearing N-terminal Gln, also exhibit behavioral abnormalities (K. Piatkov, N. Malkina, H. Wang, C. Brower and A.V., unpubl. data). Given their different N-terminal residues, Asn versus Gln, the physiological substrates of Nt^N-amidase (*Ntan1*) and Nt^Q-amidase (*Ntaq1*) are unlikely to overlap.

In *D. melanogaster*, the cleavage, by an initiator caspase, of the antiapoptotic E3 Ub ligase DIAP1 converts it into an N-end rule substrate (still active as a Ub ligase) that bears N-terminal Asn and is degraded by the deamidation branch of the Arg/N-end rule pathway^{140,141} (Fig. 10). Furthermore, an autocleavage, by the human USP1 deubiquitylase, produces a short-lived C-terminal fragment of USP1 that may be a physiological substrate of the *Ntaq1* Nt^Q-amidase²⁷⁹. A number of putative (unverified) substrates of Nt^N-amidase and Nt^Q-amidase are known to be produced by

nonprocessive intracellular proteases, including caspases (Fig. 3, Fig. 10, Section 5, and refs. 156-160).

Strong sequelogs¹ of the mammalian Ntaq1 Nt^Q-amidase are present throughout the animal phylum (including fishes, insects and nematodes), in plants, and also in some fungi, such as the fission yeast *S. pombe*⁴². Similarly, there are strong sequelogs of the mammalian Ntan1 Nt^N-amidase in other animals, and in some fungi as well. The *S. cerevisiae* Nta1 Nt^{N,Q}-amidase has sequelogs in many but not in all fungi. For example, *S. pombe* contains a sequelog of mammalian Ntaq1 but lacks a sequelog of *S. cerevisiae* Nta1. Remarkably, there is no significant sequelogy (sequence similarity¹) among Nta1, Ntan1 and Ntaq1, although all three Nt-amidases catalyze identical or similar reactions⁴². A parsimonious explanation is that a primordial Nta1 Nt^{N,Q}-amidase emerged after (not before) a split between lineages that gave rise to fungi and lineages that led to other eukaryotes. This otherwise plausible explanation is incomplete, given the presence of a sequelog of mammalian Ntaq1 in the yeast (fungus) *S. pombe* and the absence, from *S. pombe*, of a sequelog of *S. cerevisiae* Nta1.

Progress in biological research, including high-throughput structural studies, has produced a disposition all but improbable a decade ago: the mouse Ntaq1 Nt^Q-amidase, which was isolated, cloned and characterized in 2009, found itself surrounded by directly relevant genomic, proteomic and even crystallographic evidence⁴². The earlier data, produced through studies of a specific gene and its encoded 24-kDa product of unknown biochemical nature, have become unified and informed by the revealed enzymatic identity of Ntaq1, its mechanistic similarity to transglutaminases, and its place in the Arg/N-end rule pathway⁴² (Fig. 3 and Fig. 14). Specifically, a mutant, termed *tungus*, in the *D. melanogaster* Cg8253 locus, which turned out to encode Nt^Q-amidase⁴², has defective long-term memory²⁸⁰. In addition, the expression of the nematode (*C. elegans*) counterpart of mouse Ntaq1 is regulated by neuron-specific transcription factors (ref. 42 and refs. therein). These findings are consistent with behavioral abnormalities of *Ntaq1*^{-/-} mice and a major role of the Arg/N-end rule pathway in mammalian brain development²⁷¹.

The rat *Ntan1* gene, encoding Nt^N-amidase (Fig. 3), was found to be induced by ~3-fold after a 15-min exposure of rat hippocampal neurons to a static 100 mT magnetic field²⁸¹. A transient overexpression of Ntan1 in these cells (in the absence of magnetic field treatment) strongly down-regulated, through induced proteolysis, the level of Map2, a microtubule-associated protein²⁸¹. Map2 is also known to be down-regulated by magnetic fields²⁸¹. Thus Ntan1 might be involved in

degradation of Map2, through an unknown processing step that would expose the Asn residue at the N-terminus of a cleaved Map2.

9.3. N-terminal arginylation in the Arg/N-end rule pathway

The Ate1 Arg-tRNA-protein transferase (R-transferase) catalyzes the conjugation of the carboxyl group of Arg to the α -amino group of a polypeptide's N-terminal residue (Fig. 2A and Fig. 3). Strong sequelogs¹ of the ~60 kDa *S. cerevisiae* Ate1 R-transferase are present in all eukaryotes examined, including animals and plants^{26,35,36,111,112,114}. Both *S. cerevisiae* and mammalian genomes contain one *ATE1* gene, whereas plants such as *Arabidopsis thaliana* contain two *ATE1* genes, which encode highly sequelogous R-transferases^{18,113}. Mouse *Ate1* pre-mRNAs are produced, in part, from the bidirectional *Dfa*P_{*Ate1*} promoter that contains a CpG island and also expresses, in the opposite direction, a gene termed *Dfa* (divergent from Ate1)^{35,282} (Fig. 9A). *Dfa* pre-mRNAs are expressed from both *Dfa*P_{*Ate1*} and nearby promoters, are differentially spliced, and result in a complex set of mRNAs and proteins, largely though not exclusively in the testis. At least one isoform of mouse *Dfa* was shown to function, in a reporter-based *in vivo* assay, as a repressor of TATA-box transcriptional promoters²⁸². No functional or mechanistic connections between *Dfa* and R-transferase were detected so far, apart from the proximity of their head-to-head oriented genes²⁸².

In addition to the *Dfa*P_{*Ate1*} promoter, mouse *Ate1* pre-mRNAs are also produced from an upstream promoter that partially overlaps, in anti-sense direction, with ORFs encoding *Dfa* proteins^{35,282}. Six splicing-derived isoforms of the mouse Ate1 R-transferase have been identified^{26,35,112} (Fig. 9B-D). The alternatively spliced exons of these isoforms are pairs of sequelogous exons, 1A versus 1B and 7A versus 7B (Fig. 9D). Although the splicing-derived isoforms of R-transferase differ, in particular, in their substrate preferences, an unambiguously specific function of any Ate1 isoform (e.g., a distinct intracellular location or a unique *in vivo* substrate specificity) remains to be identified.

Both the mouse and *S. cerevisiae* Ate1 R-transferases are inhibited by low- μ M levels of heme (Fe³⁺-heme)³⁶. Heme binds to at least one of two conserved Heme Regulatory Motifs (HRMs) in R-transferase and inhibits its enzymatic activity through a redox mechanism that involves the formation of a disulfide bond between the vicinal Cys⁷¹ and Cys⁷² residues that are conserved among R-transferases in examined eukaryotes (Fig. 9E, F). Remarkably, heme also accelerates, *in vivo*, the degradation of mouse Ate1, thus acting as both a 'stoichiometric' and 'catalytic' down-regulator of R-transferase³⁶. Moreover, heme also binds, at least *in vitro*, to both *S. cerevisiae* and mouse Ubr1

N-recognins. Although the binding of heme to *S. cerevisiae* Ubr1 does not directly occlude any of the substrate-binding sites of Ubr1, heme blocks the allosteric activation of the third binding site of Ubr1 that recognizes an internal degron of the Cup9 transcriptional repressor³⁶. (In the absence of heme, this allosteric activation of the third binding site is caused by the occupancy of the type-1/2 sites of Ubr1 that recognize destabilizing N-terminal residues (Fig. 15).) Thus, in addition to being a sensor of NO and oxygen^{32,33}, the Arg/N-end rule pathway is also a sensor of heme (hemin) (Fig. 2A and Fig. 3). Several hemoproteins, including cytochrome oxidase and NO synthases, contain heme (Fe³⁺-heme) rather than Fe²⁺-heme as an essential prosthetic group²⁸³. Because heme interacts with physiologically relevant gases such as O₂, NO and carbon monoxide (CO), there may be a physiological connection between the NO-sensing and heme-sensing properties of the Arg/N-end rule pathway (Fig. 2A and Fig. 3). One function of this proteolytic circuit may be to coordinate the redox dynamics of heme, NO, oxygen, thiols, and other small effectors, by sensing them through components of the Arg/N-end rule pathway and by acting to alter their levels or spatiotemporal gradients, in part through conditional degradation of specific N-end rule substrates.

The inhibition of R-transferase by heme is a property that is conserved from fungi to mammals³⁶. What selective pressures led to the retention of the heme-Ate1 connection over vast phylogenetic distances? Given the cardiovascular defects of *Ate1*^{-/-} mouse embryos²⁶, their low levels of embryonic globin and heme, high levels of heme oxygenase³⁶, and their perturbed hematopoiesis (J. Sheng, R.-G. Hu and A.V., unpubl. data), the mammalian Arg/N-end rule pathway is likely to participate in the control of heme synthesis, transport, and/or catabolism. This might also be a role of Ate1-heme connections in *S. cerevisiae*. Physiological functions of Nt-arginylation in *S. cerevisiae* are still a *terra incognita*, as no defects could be detected, thus far, in *ate1Δ* yeast cells, apart from their inability to degrade N-end rule reporters with N-terminal Nd^s and Nd^l residues.

The Rgs4, Rgs5 and Rgs16 regulators of G proteins bear N-terminal Cys and are targeted for regulated degradation by the NO/O₂/Ate1-dependent branch of the mammalian Arg/N-end rule pathway^{26,32,33,41} (Sections 3 and 9.4). Rgs4 is an inhibitor of tubulogenesis, a process that underlies the development and homeostasis of blood vessels and other tubular structures, such as those in the mammary gland, kidney and lung²⁸⁴. Rgs4 and Rgs16 block signaling by the vascular endothelial growth factor (VEGF), and Rgs5 regulates vessel remodeling during neovascularization^{284,285}. As described above, heme down-regulates the Arg/N-end rule pathway *in vivo*, both through direct inhibition of the Ate1 R-transferase and through the induction of its degradation, and also (possibly)

through effects of hemin on N-recognins³⁶. In particular, hemin up-regulates Rgs4, Rgs5 and Rgs16, by inhibiting their degradation³⁶. Thus it is likely (but remains to be verified) that the Arg/N-end rule pathway is a mediator of heme's effects on tubulogenesis. The sensor-effector link between the Arg/N-end rule pathway and heme may be relevant not only to normal conditions but also to perturbations of heme homeostasis, for example upon a 'spontaneous' or wound-induced hemorrhage, or in a hemorrhage-prone setting of a growing tumor.

Heterozygous *Ate1*^{+/-} mice appear indistinguishable from their wild-type counterparts, whereas *Ate1*^{-/-} mice, which lack R-transferase and Nt-arginylation, die around embryonic day 15 (E15) with abnormalities that include cardiovascular defects²⁶. To bypass the embryonic lethality of the nonconditional absence of Nt-arginylation, the Cre-lox-tamoxifen technique was employed to induce the loss of *Ate1* in juvenile (~1 month old) *Ate1*^{+/-} mice⁴¹. The penetrance of *Ate1*^{+/-}→*Ate1*^{-/-} conversion ranged from ~90% of cells in the brain and kidney to ~60% of cells in the liver.

Remarkably, the postnatal ablation of *Ate1* caused a rapid decrease of body weight (in comparison to tamoxifen-treated control mice) and resulted in early death of ~15% of *Ate1*-deficient mice, with surviving mice attaining only ~70% of normal weight⁴¹. This failure to thrive occurred despite higher than normal food intake by *Ate1*-deficient mice. These mice contained little or no visceral fat, exhibited an increased metabolic rate, a decreased fasting blood glucose level, and an increased intestinal import and retention of amino acids and/or peptides. *Ate1*-deficient mice were also resistant to diet-induced obesity, exhibited induction of the Ucp1 uncoupling protein in white adipose tissue (WAT), and expressed strongly reduced levels of mRNA encoding proopiomelanocortin (POMC), a pituitary-produced precursor of several neurohormones with systemic and brain functions. These functions include regulation of melanocytes, a process that is perturbed in *Ate1*-deficient mice⁴¹. Because other roles of POMC-derived neurohormones include a down-regulation of food intake, a strong reduction of POMC in *Ate1*-deficient mice is consistent with their hyperphagia⁴¹. In addition, *Ate1*-deficient mice have an enhanced startle response and are strikingly hyperkinetic. They also suffer from premature kyphosis, i.e., an excessive curvature of the upper back, and from frequent seizures⁴¹. *Ate1*-deficient males are infertile, owing to defects in *Ate1*^{-/-} meiotic spermatocytes.

The remarkably broad range of biological processes that are perturbed by the loss of *Ate1* and Nt-arginylation during embryogenesis²⁶ and postnatally⁴¹ remains to be understood in mechanistic, substrate-based terms. It is virtually certain that Rgs4, Rgs5 and Rgs16, a set of regulators of G proteins that are conditionally short-lived physiological *Ate1* substrates^{32,33,36}, play a major role in

many of specific phenotypes of *Ate1*^{-/-} mice^{26,41} (Fig. 3, Fig. 10, Section 3 and Section 9.4). But other Nt-arginylation substrates are likely to be involved as well.

Studies of plant R-transferases were initiated by the identification of the *A. thaliana* mutant *delayed leaf senescence (dls1)* as a null in the *AtAte1* gene¹²⁵. Recent work, utilizing *A. thaliana* mutants lacking both *AtAte1* and *AtAte2* R-transferases and therefore completely lacking Nt-arginylation, has shown that this branch of the Arg/N-end rule pathway has a number of functions in plants, including seed germination (in part through the removal of sensitivity to the hormone abscisic acid), as well as various aspects of leave and shoot development^{18,113,126}. In particular, N-terminal arginylation was shown to play a role in repressing the meristem-promoting *BREVIPEDICELLUS (BP)* gene in developing leaves¹¹³. Most or all of these R-transferase functions require the activity of PROTEOLYSIS6 (PRT6), an E3 N-recognin Ub ligase of the plant Arg/N-end rule pathway that acts downstream of R-transferases^{113,126,273}.

Results of recent studies by the Kashina laboratory suggested that Arg might be conjugated (presumably by the *Ate1* R-transferase) to a number of different N-terminal residues of cellular proteins, i.e., that the specificity of Nt-arginylation is not confined to N-terminal Asp, Glu and (oxidized) Cys, and is determined, instead, by currently unknown internal sequence motifs in protein substrates^{127,286}. While interesting if correct, this conjecture awaits verification, because it contradicts extensive previous evidence about the substrate specificity of R-transferase (Fig. 2A and Fig. 3). A distinct kind of Arg conjugation was demonstrated to modify neurotensin, a 13-residue mammalian peptide that functions as a neurotransmitter and hormone. Arg is conjugated, via its α -amino group, to the γ -carboxyl group of an internal Glu residue of neurotensin, presumably during maturation of neurotensin precursors in the secretory pathway²⁸⁷. In the resulting 'branched' Arg-neurotensin conjugate, the orientation of the Arg moiety is opposite to that in linear, N-terminal Arg-protein conjugates that are produced by the *Ate1* R-transferase. Given this difference in chemistry and 'topology' of Arg conjugation²⁸⁷, and the apparent absence of *Ate1* from secretory compartments, it is likely (but remains to be determined) that neurotensin is arginylated by an enzyme distinct from *Ate1*.

9.4. Functions of the N-end rule pathway vis-à-vis their mechanisms

Subunit selectivity of the Arg/N-end rule pathway and degradation of a cohesin subunit.

The subunit selectivity of processive proteolysis, first discovered in the context of the Arg/N-end rule pathway^{6,13} (Fig. 11B, C), underlies most functions of the Ub system, as it makes possible

remodeling of protein complexes through subunit-selective degradation (Section 6 and Fig. 11A-D). A cleaved subunit whose C-terminal fragment bears a destabilizing N-terminal residue would be a potential substrate for the degradation-mediated removal of this subunit from a protein complex^{13,168}. Because the Arg/N-end rule pathway can target both N-degrons and specific internal degrons (Fig. 2A and Fig. 3), this remodeling can involve not only a cleaved subunit that contains an N-degron but also a full-length subunit of a protein complex. The Arg/N-end rule pathway can employ, in such settings, its capacity for subunit-specific proteolysis to reset the states of relevant circuits without destroying an entire oligomeric complex.

One example is the degradation of a separase-produced fragment of a cohesin subunit, a step that has been shown to be required for the high fidelity of chromosome cohesion/segregation^{6,28}. Although the identity of a residue at the (inferred) N-terminus of a separase-produced Scc1/Rad21 cohesin fragment varies among eukaryotes (for example, it is Arg in *S. cerevisiae*, Asn in *S. pombe*, Glu in mammals, and Cys in *D. melanogaster*)^{28,288}, the N-terminal residue of a cohesin fragment is invariably destabilizing in the Arg/N-end rule pathway (Fig. 2A, C and Fig. 6). This constraint on evolutionary alterations of the N-terminal residue is consistent with the necessity of removing, through degradation, a separase-produced cohesin fragment from the rest of cohesin, thereby ‘resetting’ the cohesin complex for reconstitution through the addition of an uncleaved Scc1/Rad21 subunit^{28,288}.

The C-terminal cohesin fragment can presumably dissociate (or be displaced) from the rest of cohesin complexes *in vivo* at a rate that allows the functioning of the cohesin machinery even in the absence of degradation of this fragment by the Arg/N-end rule pathway. But such a route is apparently inefficient enough to cause a greatly elevated frequency of chromosome loss in *ubr1Δ* *S. cerevisiae* cells and an increased genomic instability in *ate1Δ* mammalian cells. The separase-produced C-terminal fragments of Scc1/Rad21 cohesin are relatively stable proteins in both of these mutants, in contrast to their short half-lives in wild-type cells^{6,28}. It has also been shown that at least in yeast it is specifically the metabolic stabilization of the Scc1 cohesin fragment (as distinguished from other effects of the absence of the Arg/N-end rule pathway) that increases the frequency of chromosome loss²⁸. Another example of subunit-selective proteolysis is a circuit that regulates the import of peptides through the degradation of Cup9, a transcriptional repressor of the peptide transporter Ptr2. The *S. cerevisiae* Arg/N-end rule pathway targets Cup9, through its internal degron, for a subunit-selective, conditional degradation. Specifically, the short-lived Cup9 is a part of

a repressor complex that also contains the more abundant and longer-lived Ssn6-Tup1 global repressor^{27,138} (Fig. 15).

Regulation of G proteins and the Arg/N-end rule pathway. As mentioned in Sections 3, 5 and 9.3, the functions of the mammalian Arg/N-end rule pathway include the sensing of NO and oxygen, and the regulation of specific G proteins that are coupled to transmembrane receptors. These processes involve the NO/O₂-dependent degradation of Rgs4, Rgs5 and Rgs16, a set of G-protein regulators^{32,33,36} (Fig. 3 and Fig. 10). The conditional Nt-arginylation and degradation of these RGS proteins by the Arg/N-end rule pathway alters the activity of cognate G-protein circuits^{32,33}. In particular, it has been shown that an induction of angiogenesis specifically in the heart results in a strong myocardial hypertrophy (through enlargement of cardiomyocytes), apparently because an increased density of the heart's vascular bed augments the levels of NO that is produced by endothelial cells²⁸⁹. If so, this effect of increased NO on cardiomyocytes is likely to be mediated, at least in part, through an acceleration of the NO/O₂-dependent degradation of Rgs4 (as well as Rgs5 and Rgs16) by the Arg/N-end rule pathway in NO-exposed cardiomyocytes. Indeed, the inhibition of G_q-protein signaling by Rgs4 is known to be a negative modulator of the myocardial hypertrophic response (ref. 289 and refs. therein). In sum, the Arg/N-end rule pathway is likely to be a major regulator of the heart. Recent studies indicated that the rate of degradation of Rgs4, largely by the Arg/N-end rule pathway (Fig. 3), influences other physiological and pathophysiological processes as well, including the invasiveness of breast cancer, responses of neurons to opiates, and responses of cells in culture to fluxes of calcium ions^{290,291}.

The Cre recombinase-induced postnatal loss of the mouse Ate1 R-transferase and Nt-arginylation (Section 9.3) leads to a more than 10-fold increase in the level of the metabolically stabilized Rgs4 in Ate1-deficient mice, suggesting a major decrease in signaling by Rgs4-regulated G proteins⁴¹. Although the loss Nt-arginylation does result in a number of phenotypic alterations in Ate1-deficient mice (Section 9.3), it is remarkable that the strikingly strong effect of this loss on the levels of several RGS proteins^{32,41} is still compatible with most physiological functions. This robustness implies a compensatory feedback regulation, which remains to be explored.

Regulation of apoptosis and the Arg/N-end rule pathway. As mentioned in Section 9.2, one example of involvement of the Arg/N-end rule pathway in the control of apoptosis is the conditional degradation of *D. melanogaster* DIAP1, a major antiapoptotic regulator^{140,141} (Fig. 10). A functional understanding of DIAP1 circuits vis-à-vis the Arg/N-end rule pathway remains to be attained. Some

C-terminal fragments of other proteins that are cleaved by activated initiator and/or effector caspases are either previously demonstrated or putative (predicted) N-end rule substrates (Section 5 and Fig. 10). Such fragments bear destabilizing N-terminal residues and would be expected to be targeted for degradation by the Arg/N-end rule pathway (refs. 6,140,141,146,147 and refs. therein). If the cleavage of a protein by, for example, an initiator caspase produces a proapoptotic C-terminal fragment, a destabilizing N-terminal residue and (therefore) a short *in vivo* half-life of such a fragment would counteract the induction of apoptosis by spuriously activated caspases that are present even in unperturbed cells, given the level of noise in caspase-activation circuits. This antiapoptotic role of the Arg/N-end rule pathway was recently found to be significant not only in relatively unstressed cells but under proapoptotic conditions as well (K. Piatkov, C. Brower and A.V., unpubl. data).

Human immunodeficiency virus-1 (HIV-1) integrase and the Arg/N-end rule pathway.

During infection by the human immunodeficiency virus (HIV), the viral RNA genome is reverse-transcribed, and the resulting cDNA is inserted into the chromosomal DNA of infected mammalian cells. This process is mediated by HIV integrase, in association with viral and host proteins. The integrase is a member of the family of structurally related DNA transferases that includes phage transposases and mammalian VDG recombinases RAG1 and RAG2. The HIV integrase is initially a part of the HIV Gag-Pol polyprotein that resides in the virion. The integrase is excised from the polyprotein by the viral protease while still in the virion, and is released into the cytosol upon infection. At this stage, the excised integrase bears N-terminal Phe, an Nd^p residue in the Arg/N-end rule pathway (Fig. 3 and Fig. 10), and has been shown to be targeted by this pathway for degradation^{132,133} ($t_{1/2} < 30$ min). In contrast, the otherwise identical integrase bearing, for example, N-terminal Met, was significantly longer-lived *in vivo*¹³².

Mutant HIV-1 viruses in which the N-terminal residue of (excised) integrase was changed from Phe to Met were viable but impaired (~5-fold or more) in replication and infectivity, in comparison to otherwise identical viruses containing Phe-integrase¹³³. This impairment included defects in early phases of infection, particularly reverse transcription and integration of the viral cDNA. Control experiments have shown that the impairment was not caused by a lower efficiency of Met-integrase excision from the polyprotein, in comparison to the excision of Phe-integrase¹³³. Specific mechanisms through which a metabolic stabilization (and thus higher steady-state levels) of integrase perturb both reverse transcription and integration remain to be understood. One possibility

is that an excess of HIV-1 integrase might inhibit reverse transcription through the known physical interaction between the integrase and reverse transcriptase¹³³. The cited studies were carried out before the discovery of the Ac/N-end rule pathway³. Therefore, although the replacement of N-terminal Phe of the HIV-1 integrase with N-terminal Met stabilized the resulting protein¹³², it remains to be determined whether N-terminal Met, an Nd^s destabilizing residue in the Ac/N-end rule pathway (Fig. 2B), was Nt-acetylated in the posttranslationally formed Met-integrase (N-terminal sequence Met-Leu-Asp) after its release into the cytosol, and if so whether Met-integrase was targeted for degradation by the Ac/N-end rule pathway.

Many viruses encode polyproteins that yield, upon excision of individual proteins by viral proteases, either putative or confirmed N-end rule substrates. One example (in addition to HIV-1 integrase), is the polyprotein-derived nsP4, the RNA polymerase of the Sindbis virus. This enzyme has N-terminal Tyr (an Nd^p residue in the Arg/N-end rule pathway) (Fig. 3), is metabolically unstable, and has been shown to be partially stabilized by replacements of its N-terminal Tyr by the Met or Ala residues¹³¹.

Human birth defects and the N-end rule pathway. Johanson-Blizzard syndrome (JBS) is an autosomal recessive disorder caused by mutational inactivation of both copies of the *UBR1* gene that encodes an N-recognin³⁴. JBS includes a congenital exocrine pancreatic insufficiency and inflammation, multiple malformations (such as nasal wing aplasia), as well as frequent mental retardation and deafness. The severity of JBS symptoms is decreased if at least one of the mutant copies of *UBR1* encodes a partly functional missense hypomorph of the UBR1 E3 Ub ligase (C.-S. Hwang, M. Zenker and A.V., unpubl. data). *Ubr1*^{-/-} mice²⁹² exhibit milder versions of human JBS symptoms, including exocrine pancreatic insufficiency³⁴. The mechanistic cause(s) of JBS remains to be understood, in part because all other UBR-type N-recognins (Section 9.1), including UBR2, a strong sequelog of UBR1 that is expressed in exocrine pancreas as well, are retained in JBS patients. Their cells, therefore, still contain the Arg/N-end rule pathway (Fig. 3). It is possible that UBR1, despite being 47% identical to UBR2^{30,292}, has a JBS-relevant physiological substrate(s) that is confined to UBR1. In addition, previous work has shown that *S. cerevisiae* Ubr1 is an activity-limiting component of the yeast Arg/N-end rule pathway¹². Thus it is also possible that some JBS phenotypes may be caused by a suboptimal overall activity of the Arg/N-end rule pathway in JBS patients, as distinguished from the loss of a function unique to UBR1.

Lyon and colleagues have recently identified the first human birth defect in the Ac/N-end rule pathway (Fig. 2B), an X-linked infantile lethal disorder caused by a hypomorphic missense mutation in *NAA10* (*hARD1*), which encodes the catalytic subunit of the NatA Nt-acetylase (G. J. Lyon, a personal communication). This enzyme Nt-acetylates the N-terminal Ala, Ser, Thr, Val, Cys and Gly residues of nascent proteins after the cotranslational removal of their N-terminal Met by Met-aminopeptidases^{91-94,102}. The early postnatal lethality of the human NAA10 syndrome is consistent with lethality of null *NAA10* mutants in *Trypanosoma brucei*, *C. elegans* and *D. melanogaster* (refs. 293,294 and refs. therein). Given the necessity of Nt-acetylation for the activity of ^{Ac}N-degrons³, an increased fraction of non-Nt-acetylated physiological substrates of NatA in human or other hypomorphic *NAA10* mutants would be expected to (at least) partially stabilize these proteins against degradation. Detrimental effects of this stabilization (Section 8) may be one cause of the early postnatal lethality of the human NAA10 syndrome, despite a significant residual activity of the mutant NAA10 Nt-acetylase (G. J. Lyon, a personal communication).

Regulation of bacterial virulence and the Arg/N-end rule pathway. *Listeria monocytogenes* is a gram-positive bacterial pathogen of humans and other animals. Following internalization into mammalian cells, bacteria are initially contained within host vacuoles but rapidly escape into the cytosol (where they grow and divide), largely through the perforation of the phagosomal membrane by the pore-forming toxin called listeriolysin O (LLO). LLO that is secreted from bacterial cells into the cytosol of mammalian cells bears N-terminal Lys, an Nd^P residue (Fig. 3 and Fig. 10). LLO is indeed a short-lived N-end rule substrate in mammalian cells¹²⁴. Moreover, replacement of N-terminal Lys of LLO with Val not only increased the *in vivo* half-life of LLO but also decreased the virulence of *L. monocytogenes*¹²⁴. Thus the presence of strongly destabilizing residues at the N-termini of LLO toxins from *L. monocytogenes* and related bacteria¹²⁴ is likely to have been selected, during evolution, to optimize, through down-regulation, the levels of secreted LLO in the cytosol of infected mammalian cells.

Regulation of DNA repair and the Arg/N-end rule pathway. O⁶-methylguanine (O⁶meG) and related modifications of guanine in double-stranded DNA are lesions that can be produced by many alkylating agents, including N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a potent carcinogen. O⁶meG is repaired through demethylation by the O⁶-methylguanine-DNA alkyltransferase (AGT). This protein is called MGMT in mammals and Mgt1 in *S. cerevisiae*. AGT proteins remove methyl and other alkyl groups from an alkylated O⁶ in guanine by transferring the adduct to an active-site

Cys residue (ref. 40 and refs. therein). The resulting S-alkyl-Cys of AGT is not restored back to Cys, so repair proteins of this kind can act only once. Recent work has shown that *S. cerevisiae* Mgt1 (preferentially but not exclusively its S-alkylated form) is targeted for polyubiquitylation and degradation by the Arg/N-end rule pathway, specifically by its double-E3 Ubr1/Rad6-Ufd4/Ubc4 targeting complex^{40,44} (Fig. 2A and Section 9.1). The Ubr1/Ufd4-dependent Arg/N-end rule pathway mediates not only the constitutive but also MNNG-accelerated degradation of Mgt1. Although the degradation signal of Mgt1 is near its N-terminus, it is not an N-degron^{40,44}.

Because the Ubr1 and Ufd4 E3s physically interact, it is remarkable that the degron of Mgt1 can be recognized, independently, by either Ubr1 or Ufd4, possibly in a mutually exclusive manner, a previously undescribed mode of 'alternative' degron recognition that remains to be understood both functionally and mechanistically (ref. 40; A. Shemorry, C.-S. Hwang and A.V., unpubl. data). It is also unknown whether the targeting and polyubiquitylation of Mgt1 by the Ubr1/Rad6-Ufd4/Ubc4 complex can involve, *in vivo*, the DNA-bound Mgt1 protein or whether this targeting requires a dissociation of Mgt1 from DNA. *A priori*, it is likely (nothing is known about this at present) that Mgt1 functions as a part of a chromosome-associated protein complex. If so, degradation of Mgt1 may involve the subunit selectivity of the Arg/N-end rule pathway¹³, an aspect of its activity that is also essential for selective degradation of the separase-produced C-terminal fragment of the Scc1 cohesin subunit that is bound to the rest of cohesin complex²⁸. In both cases, the alkylated (i.e., irreversibly inactivated) Mgt1 and the fragment of cohesin subunit are obligatory 'dead-end' products of their respective circuits that are still parts of larger complexes. As discussed above in the context of cohesin, the Arg/N-end rule pathway operates, in these settings, as a homeostatic device that employs its capacity for subunit-selective proteolysis to reset the states of relevant circuits. It is likely (but remains to be determined) that MGMT, the mammalian counterpart of yeast Mgt1, is also regulated through degradation by the Arg/N-end rule pathway. The activity of MGMT in repairing alkylated DNA is of major relevance to the efficacy of DNA-alkylating anticancer drugs, a set of cytotoxic compounds extensively employed in cancer therapy²⁵⁵.

Regulation of peptide transport and the Arg/N-end rule pathway. Peptides can serve as a source of amino acids and nitrogen in all organisms. The import of di- and tripeptides (di/tripeptides) in *S. cerevisiae* is controlled by the Arg/N-end rule pathway^{295,296} through a specific design of the Ubr1 E3 Ub ligase. The type-1/2 substrate-binding sites of Ubr1 bind to the type-1 and type-2 destabilizing N-terminal (Nd^p) residues of proteins or short peptides, whereas the third binding site of

Ubr1 recognizes an internal degron of Cup9, a homeodomain transcriptional regulator, largely a repressor, of more than 30 genes^{38,138}. The regulon of Cup9 includes *PTR2*, a gene encoding the main importer of di/tripeptides^{297,298}. The binding of imported di/tripeptides bearing destabilizing N-terminal residues to the type-1/2 sites of Ubr1 allosterically activates the otherwise autoinhibited Cup9-binding site of Ubr1. The resulting increase in the fraction of Ubr1 molecules that can target Cup9 for polyubiquitylation leads to a faster degradation of Cup9 ($t_{1/2} < 2$ min) and thereby causes a derepression of the *PTR2* gene. This positive-feedback circuit allows *S. cerevisiae* to detect the presence of extracellular peptides and to react by increasing the rate of their uptake^{27,29,138} (Fig. 15). Genes of the Cup9 regulon other than *PTR2* that are also induced by di/tripeptides include additional components of the peptide transport and utilization system, for example *OPT2*, which encodes an oligopeptide importer, and also peptidases that process imported peptides to amino acids^{297,299}.

The properties of *S. cerevisiae* Ubr1 that underlie the regulation of peptide import through the conditional degradation of Cup9 were found to require phosphorylation of Ubr1 at Ser³⁰⁰ by the sequelogous type-I casein kinases Yck1 and Yck2 (Fig. 7A, B and Fig. 15). Interestingly, the phosphorylation of Ubr1 at Ser-300 by Yck1/2 initiates ('primes') a specific phosphorylation cascade that results in phosphorylation of Ser²⁹⁶, Ser²⁹², Thr²⁸⁸, and Tyr²⁷⁷ of Ubr1 by Mck1, a kinase of the glycogen synthase 3 (Gsk3) family³⁹ (Fig. 7A, B). In contrast to the functionally critical phosphorylation at Ser³⁰⁰ by Yck1/2, the subsequent phosphorylations of Ubr1 by the Mck1 kinase had at most minor effects on the known properties of Ubr1, including regulation of peptide import³⁹. A function of Ubr1 that requires the Mck1-mediated phosphorylation cascade (Fig. 7A, B) remains to be discovered.

The induction of the Ptr2 transporter by di/tripeptides, a process controlled by the Arg/N-end rule pathway (Fig. 15), is just one of regulatory inputs that couple Ptr2 expression to the availability and quality of nutrients. For example, Ptr2 expression is down-regulated by certain nitrogen sources, including ammonia, but not by other nitrogen sources, such as urea or allantoin³⁰⁰. Several systems, including the Arg/N-end rule pathway, ensure that a cell does not waste resources synthesizing large amounts of the Ptr2 transporter in the absence of extracellular peptides, or when a more efficacious nitrogen source, such as ammonia, is present. Ptr2 is induced not only by short peptides but also by extracellular amino acids, particularly Leu and Trp. This response would be adaptive in natural habitats, because amino acids that *S. cerevisiae* (a scavenging heterotroph) encounters outside a laboratory tend to be products of protein breakdown and thus signify a likely presence of short

peptides. It has also not been precluded that Ptr2 might import some amino acids, in addition to peptides.

Extracellular amino acids regulate *S. cerevisiae* Ptr2 through the SPS (*SSY1-PTR3-SSY5*) system³⁰¹⁻³⁰⁴. Ssy1 is an integral membrane protein and sensor of amino acids that does not function as a transporter. Ssy1 'measures' the concentration ratio of an amino acid across the plasma membrane and signals through the rest of the SPS pathway to induce a regulon that includes a number of amino acid transporters as well as the Ptr2 peptide transporter. This signaling employs a proteolytic activation of latent (conditionally cytosolic) transcriptional activators Stp1 and Stp2, leading to their import into the nucleus and the induction of the SPS regulon³⁰¹⁻³⁰⁴. Our recent work has shown that an extracellular amino acid such as Trp acts via the SPS system to induce the Ptr2-mediated import of di/tripeptides not only through activation of positive transcriptional regulators such as Stp1/2 but also through acceleration of the degradation of the Cup9 repressor by the Arg/N-end rule pathway¹³⁸ (Fig. 15).

It is likely that the Arg/N-end rule pathway regulates peptide transport in multicellular eukaryotes as well. In contrast to *S. cerevisiae*, mammalian cells contain peptide transporters not only in the plasma membrane but also in the ER membrane. These ER proteins, called TAP transporters, mediate the import of peptides (including peptides produced by the proteasome-mediated degradation of cytosolic and nuclear proteins) from the cytosol into the lumen of ER, where these peptides are loaded onto MHC class I proteins en route to the cell surface, for presentation of MHC-peptide complexes to T lymphocytes³⁰⁵. Might a mammalian peptide-sensing circuit based on the Arg/N-end rule pathway regulate the expression of TAP transporters as well? This and related questions remain to be addressed.

Results of a recent study suggested that the mammalian Arg/N-end rule pathway plays a role, through the Ubr1 and Ubr2 N-recognins, in the control of protein synthesis by amino acids³⁰⁶. Specifically, mammalian Ubr1 and Ubr2 appear to recognize (bind to) free Leu (ref. 306), as distinguished from the binding of these N-recognins to dipeptides bearing N-terminal Leu. Such dipeptides bind to, in particular, *S. cerevisiae* Ubr1 with K_d in a low- μ M range³⁸ (Section 9.1). In contrast, *S. cerevisiae* Ubr1 has a considerably (more than 10-fold) lower affinity for free amino acids³⁸. Therefore it remains to be determined, using quantitative binding assays that involve free (non-immobilized) Leu, whether its affinity for mammalian Ubr1 is in a range that would be likely to be relevant physiologically. The possibility of Ubr1/2 involvement in the control of protein synthesis

was also suggested by the finding that overexpression of rat Ubr1 and Ubr2 in rat cells inhibited the activity of mTOR kinase, a key regulator of translation, and that the inhibition could be reversed by millimolar levels of Leu in the medium³⁰⁶.

The N-end rule pathway and degradation of misfolded proteins. Errors in specific steps of protein synthesis result, conservatively, in 5% to 20% of molecules of a 50 kDa protein containing at least one missense alteration that is not encoded by DNA^{307,308}. Premature termination of translation and frameshifts are additional sources of defective polypeptides. The misfolding and aggregation of mistranslated proteins and the resulting toxicity are significant even in the absence of environmental insults³⁰⁷⁻³¹¹. Recent studies have shown that Ubr1, the E3 N-recognin of the *S. cerevisiae* Arg/N-end rule pathway, is a part of quality-control systems that function to selectively eliminate misfolded proteins in the nuclear and cytosolic compartments¹¹⁹⁻¹²¹. In yeast, these systems include the import (assisted by chaperones of the Hsp70 family) of misfolded cytosolic proteins into the nucleus, followed by their degradation there by pathways that involve both the San1 E3 Ub ligase and the Ubr1-mediated Arg/N-end rule pathway^{120,121,312-314}. UBR-type N-recognins of the mammalian Arg/N-end rule pathway were also found to play a role in degradation of misfolded proteins¹²².

Does the *S. cerevisiae* Ubr1 N-recognin mediate the degradation of misfolded proteins as the double-E3 Ubr1-Ufd4 complex (Fig. 2A), or does it, perhaps, interact with other, 'alternative' E3s as well? Does the targeting of misfolded proteins by Ubr1 involve N-degrons that would be produced through preliminary cleavages of misfolded proteins by (nonprocessive) proteases, or are such proteins targeted by the Arg/N-end rule pathway through their internal degrons, exposed as a result of misfolding? If so, are these degrons recognized by the substrate-binding sites of Ubr1 that target the internal degrons of the Cup9 repressor^{29,38} or the Mgt1 DNA repair protein⁴⁰, or does the targeting of misfolded proteins involve other, currently unknown binding sites of Ubr1? Does the *in vivo* degradation of some misfolded proteins also involve the Ac/N-end rule pathway and ^{Ac}N-degrons³ (Fig. 2B)? These cotranslationally formed N-degrons are present in a majority of cellular proteins, and were suggested to mediate the selective elimination of unassembled, misfolded or otherwise aberrant proteins³ (Section 8). If ^{Ac}N-degrons contribute to selective destruction of misfolded proteins, might some of the E3s involved that are presumed, at present, to function outside of the Ac/N-end rule pathway, have binding sites for ^{Ac}N-degrons? As illustrated by these questions, our understanding of the Arg/N-end rule and Ac/N-end rule pathways as components of protein quality control is just beginning.

The roles of the N-end rule pathway described above are but a small subset of its currently known functions, summarized in Section 3. Many of these functions were discovered through genetic approaches and therefore remain to be understood in mechanistic, substrate-based terms.

10. Prokaryotic N-end rule pathways

Although prokaryotes (bacteria and archaea) lack a *bona fide* Ub system, at least gram-negative bacteria contain a Ub-independent version of the N-end rule pathway, termed the Leu/N-end rule pathway (Sections 3 and 5, Fig. 5 and Fig. 6). The Leu/N-end rule pathway was discovered in 1991 (ref. 14), and was characterized in gram-negative bacteria *E. coli* and *Vibrio vulnificus*^{16,37}. It comprises the following components^{14,16,19,37,71-79,81,186,188,189,191,199,315-318}. (1) ClpAP, a proteasome-like, ATP-dependent protease; (2) ClpS, the 12-kDa N-recognin of the Leu/N-end rule pathway that binds to N-terminal Leu, Phe, Trp or Tyr (the pathway's Nd^P residues) and delivers N-end rule substrates to the ClpAP protease (Fig. 5 and Fig. 6B-D); (3) Aat, an L/F-transferase that employs Leu-tRNA or Phe-tRNA as a cosubstrate to conjugate largely Leu (and occasionally Phe) to the N-termini of proteins bearing N-terminal Lys or Arg, the secondary destabilizing (Nd^S) residues of the Leu/N-end rule pathway (in contrast, Lys and Arg are Nd^P residues in eukaryotes; Fig. 2A; *cf.* Fig. 5). Crystal structures of Aat suggested a catalytic mechanism of this enzyme³¹⁶⁻³¹⁸; (4) Bpt, an L-transferase that employs Leu-tRNA to conjugate Leu to N-terminal Asp, Glu, and (possibly) oxidized Cys (Fig. 5B).

V. vulnificus contains both the Aat and Bpt L-transferases, but *E. coli* contains only Aat. Therefore N-terminal Arg, Lys, Asp and Glu are secondary destabilizing (Nd^S) residues in *V. vulnificus*, whereas in *E. coli* the N-terminal Asp and Glu are stabilizing ('non-destabilizing') residues³⁷ (Fig. 5). In *V. vulnificus*, the two L-transferases are encoded by the *aat-bpt* operon, but in several other gram-negative bacteria these genes are unlinked, and some gram-negatives lack one or the other of these L-transferases (for example, *E. coli* lacks Bpt)³⁷. Genomes of examined gram-negative bacteria encode ClpS, but archaea (archaeobacteria) and gram-positive bacteria appear to lack significant sequelogs of Aat, Bpt, and ClpS, and thus may lack the Leu/N-end rule pathway. This is a tentative conclusion, because, to the best of my knowledge, no biochemical tests, using N-end rule reporter proteins, for the presence of a Leu/N-end rule pathway or its analog have been carried out, thus far, with a gram-positive bacterium or an archaeon.

The ~27-kDa bacterial Bpt L-transferases are sequelogenous to the ~60-kDa eukaryotic Ate1 R-transferases (Figs. 6A), strongly suggesting their homology, i.e., a common ancestry in evolution. In contrast to Ate1 R-transferases, Bpt L-transferases contain the CP...CXXCXXC motif (Fig. 6A; X is any residue; in some Bpt enzymes, Pro is replaced by Ser or other residues). This set of sequences is one of consensus motifs for binding a complex of nonheme iron and inorganic sulfide called a Fe-S cluster. Such complexes are present in many proteins and mediate a broad range of biological processes, including respiration, photosynthesis, redox catalysis, gene regulation, and a variety of enzymatic reactions^{319,320}. At least some cysteines of the CP...CXXCXXC motif are required for the activity of the *V. vulnificus* Bpt L-transferase. The presence of this motif (Fig. 6A) and spectroscopic properties of isolated Bpt are consistent with it being a Fe-S cluster protein (H. Wang and A. V., unpubl. data).

Specific mechanisms of the ClpS/ClpAP-mediated targeting and degradation of N-end rule substrates by the bacterial Leu/N-end rule pathway^{16,75,76,78,82} are described in Section 5 (Fig. 12). These mechanisms are understood in greater detail than the analogous but Ub-dependent targeting in the eukaryotic Arg/N-end rule pathway (Section 5, Fig. 2A, Fig. 3 and Fig. 11). A sequelogy (sequence similarity) between the substrate-binding segment of the 12-kD bacterial ClpS N-recognin and the functionally analogous segment of a vastly larger E3 Ub ligase such as the yeast or mammalian Ubr1 N-recognin (Fig. 6D) suggests homology, i.e., a common descent of bacterial and eukaryotic N-recognins (Section 12).

Two physiological substrates of the Leu/N-end rule pathway have recently been discovered^{76,77}. One of them is Dps, an 18-kDa DNA-binding protein that compacts the nucleoid of *E. coli* in starving cells, forming highly ordered, crystal-like structures. An unknown protease removes 5 residues from the initial N-terminus of Dps, producing N-terminal Leu (an Nd^p residue) and thereby making Dps a short-lived N-end rule substrate. In a plausible model, a protease that produces the N-degron of Dps may do so in a regulated manner, at a time when the bulk of Dps should be removed from DNA through processive degradation by the Leu/N-end rule pathway. Remarkably, Dps can also be destroyed as the full-length (uncleaved) protein, by another processive protease, ClpXP, which targets the N-terminus-proximal segment of Dps. This segment is at least partially removed by a protease that produces the N-degron of Dps. An interplay between ClpS-dependent and ClpS-independent degradation of Dps, and physiological ramifications of this circuit remain to be understood^{76,77}.

The other identified N-end rule substrate is the *E. coli* YgjG putrescine-aminotransferase (PATase). It is targeted for degradation by the Leu/N-end rule pathway through a route that requires Nt-leucylation by the Aat L/F-transferase^{76,77} (Fig. 5A). The evidence by Ninnis *et al.*⁷⁷ suggests that Aat can conjugate Leu or Phe to the (initially present) N-terminal Met residue of PATase, as distinguished from the previously characterized specificity of Aat, which conjugates (preferentially) Leu to N-terminal Arg or Lys of N-end rule substrates. It is possible that the active site of the Aat L/F-transferase might be able to accommodate the N-terminal Met of a substrate for its conjugation to Leu if the substrate's second residue is, for example, Asn, a small hydrophilic residue⁷⁷. It remains to be determined whether Aat can Nt-leucylate, *in vivo*, some N-terminal motifs that contain N-terminal Met. Physiological roles of the degradation of the putrescine-catabolizing PATase by the Leu/N-end rule pathway, e.g., in controlling PATase levels as a function of *in vivo* putrescine levels^{76,77}, remain to be addressed as well.

Both gram-negative and gram-positive bacteria employ the Nt-formylated Met residue (fMet) to initiate the synthesis of a polypeptide chain. The resulting N-terminal fMet of nascent bacterial proteins is cotranslationally deformylated by a ribosome-bound deformylase³²¹. In contrast to bacteria and similarly to eukaryotes, archae initiate translation using unmodified Met, and in addition cotranslationally Nt-acetylate a large subset of their proteins³²². The protein $\alpha 1$ is one of two subunits that form the 20S core proteasome in the archaeal prokaryote *Haloferax volcanii*. Interestingly, the *in vivo* concentration of $\alpha 1$ was found to depend on the identity of a second-position residue that follows the N-terminal Met of the $\alpha 1$ protein³²³. In particular, the levels of N-terminal mutants of the $\alpha 1$ subunit that were partially non-Nt-acetylated were strikingly higher than the levels of Nt-acetylated (wild-type) $\alpha 1$, in the absence of increased expression of $\alpha 1$ mRNA³²³. Although the authors did not consider the possibility of ^{Ac}N-degrons, their findings with *H. volcanii* $\alpha 1$ and its mutants may be accounted for by the presence of the Ac/N-end rule pathway in archaeal prokaryotes. This possibility remains to be examined.

11. Mitochondrial N-end rule pathway

Mitochondria of eukaryotic cells are descendants of gram-negative bacteria, which contain the Leu/N-end rule pathway (Fig. 3B, C and Section 9). Might the inner matrix of mitochondria (a counterpart of bacterial cytosol) also contain an N-end rule pathway?⁵ Recent results by Pfanner, Meisinger, Vögtle and their colleagues did reveal such a pathway^{277,278,324}. Specifically, most

N-terminal presequences of nuclear-encoded inner-matrix mitochondrial proteins are cleaved off by the mitochondrial processing protease (MPP) upon the import of these proteins from the cytosol into the matrix. The cleavage specificity of mitochondrial MPP (it is similar to that of the signal peptidase in the ER membrane) often results in N-terminal residues of processed proteins that are destabilizing in both the bacterial Leu/N-end rule pathway and the eukaryotic (cytosolic/nuclear) Arg/N-end rule pathway (Fig. 2A, Fig. 4B and Fig. 5). Vögtle *et al.*²⁷⁷ identified an aminopeptidase, termed Icp55, in the mitochondrial matrix of *S. cerevisiae* that removes a single (bulky hydrophobic) destabilizing residue from the N-termini of matrix-imported, MPP-processed mitochondrial proteins. This processing step tends to yield proteins that bear unmodified N-terminal residues such as Ala, Ser or Thr. Remarkably, some of the normally long-lived mitochondrial-matrix proteins become short-lived in *icp55Δ* cells, whose mitochondria lack the Icp55 protease²⁷⁷.

Thus at least the bulky hydrophobic N-terminal residues, which are destabilizing in both the Arg/N-end rule pathway and the bacterial Leu/N-end rule pathway (Fig. 4B), are destabilizing in mitochondria as well, except that in wild-type cells these residues are cleaved off, by Icp55, from the N-termini of imported matrix proteins. One function of the Icp55 aminopeptidase is apparently to reduce or preclude the degradation of newly imported mitochondrial proteins by an N-end rule pathway in this organelle^{277,278}. Yet another mitochondrial processing peptidase, Oct1, also contributes, in addition to Icp55, to modifying the N-termini of specific proteins that are imported into the matrix, so that their mature N-terminal residues are not destabilizing in the bacterial-type N-end rule³²⁴. In *oct1Δ* yeast cells, the substrates of the Oct1 protease retain destabilizing N-terminal residues (Fig. 4B and Fig. 5) and are short-lived in the inner matrix³²⁴.

Mitochondria presumably ‘inherited’ their N-end rule pathway from their bacterial ancestors. It is likely that processing proteases such as Icp55 and Oct1 evolved as ‘suppressors’ that prevent the degradation of otherwise short-lived mitochondrial N-end rule substrates that form upon the import of proteins from the cytosol into the matrix. If so, what exactly is the function of the N-end rule pathway in mitochondria? In particular, what mitochondrial proteins remain physiological N-end rule substrates in the presence of Icp55 and Oct1, which ‘rescue’ specific proteins from becoming substrates of the N-end rule pathway? What is the identity of an N-recognin and a processive ‘downstream’ protease that recognize and degrade N-end rule substrates in mitochondria? These questions remain to be addressed. Because yeast Icp55 and Oct1 are evolutionarily conserved proteases, an N-end rule pathway is likely to be present in mitochondria of all eukaryotes.

Remarkably, putative counterparts of mitochondrial Icp55 are also present at least in the cytosol²⁷⁷. Thus a previously unexplored possibility is that some cytosolic/nuclear substrates of the Arg/N-end rule pathway may be subject to N-terminal 'editing' (and thus a rescue from degradation) by specific aminopeptidases that would act, in such settings, similarly to the mitochondrial Icp55 and Oct1 proteases.

Chloroplasts of plant cells are descendants of ancient cyanobacteria, which may have also contained an N-end rule pathway. Indeed, chloroplasts (but apparently not mitochondria) contain a strong sequelog of bacterial ClpS N-recognins (Fig. 6D), suggesting a chloroplast Leu/N-end rule pathway. This possibility remains to be verified.

12. Evolution of the N-end rule pathway

An N-terminal residue is present even in dipeptides. Thus N-degrons of polypeptide substrates were available as targets of proteolytic systems from early stages of protein evolution, in agreement with the presence of N-end rule pathways in both prokaryotes and eukaryotes. Primordial polypeptides, produced by ribozymes that eventually became ribosomes^{325,326}, were at first (most likely) short, disordered and therefore prone to aggregation or other noncognate interactions. Thus damage control pathways that involve chaperones and selective proteolysis^{182,327} were probably as vital at the dawn of protein-based life as they are in extant cells. Prior to emergence of quasi-modern proteins, primordial proteases and chaperones consisted, at least in part, of specific RNAs or other nucleic acids. Over ensuing eons, a succession of changes, driven by selection for cells that produced optimal amounts of fitness-increasing polypeptides, led to the emergence of the genetic code, ribosomes and the rest of the translation apparatus. This evolution also led to DNA-encoded polypeptides that began to resemble their modern counterparts, including functional properties of specific proteins and their ability to stay in solution. Fundamental similarities among the extant bacteria, archaea and eukaryotes indicate that the last universal common ancestor (LUCA) of modern organisms was a cell that expressed a broad range of proteins^{326,328-330}. LUCA also contained proteolytic systems, including, probably (for the reasons above), an N-end rule pathway.

The Nt-acetylation of cellular proteins apparently predated LUCA, as both bacteria, archaea and eukaryotes contain Nt-acetylases^{91,92}. Thus a version of the Ac/N-end rule pathway³ (Fig. 2B) may have already been in place by the time of LUCA. As to the Leu/N-end rule and Arg/N-end rule pathways, their presence in Gram-negative bacteria and in eukaryotes, respectively (Fig. 2A and

Fig. 5), suggests an emergence of a version of the Leu/N-end rule pathway in pre-LUCA cells. The N-terminal Leu, Phe, Tyr and Trp residues of N-end rule substrates are recognized both by the 12-kDa *E. coli* ClpS and by the type-2 binding site of eukaryotic N-recognins such as the 200 kDa human UBR1 E3 Ub ligase (Fig. 6B-D). The highly sequelogous segments in the substrate-binding sites of bacterial ClpS and eukaryotic Ubr1 suggests a common ancestry for these otherwise nonsequelogenous proteins of vastly different sizes^{72,74,83-85,331} (Fig. 6D), presumably through the emergence, at first, of a ClpS-like protein in a lineage of cells that led to LUCA.

LUCA might have also contained early versions of Bpt and/or Aat L-transferases, which act upstream of ClpS in the Leu/N-end rule pathway (Fig. 5). After separation of prokaryotic and eukaryotic lineages, the expanding rule books of the N-end rule continued to be similar in all organisms (Fig. 4B), but the underlying machinery began to diverge. In particular, Ub ligases and deubiquitylases have emerged (or, alternatively, were retained) only in eukaryotes^{37,332}. Some bacteria express mediators or inhibitors of the Ub system that are injected into eukaryotic hosts during infection, but these proteins do not seem to have Ub-related functions in bacteria themselves³³³⁻³³⁷. (Possible causes of the absence of a *bona fide* Ub system in extant prokaryotes are considered in Section 5.) In addition, the problem of recognizing basic and acidic (as distinguished from bulky hydrophobic) N-terminal residues in later versions of the N-end rule pathway was solved quite differently in eukaryotes versus bacteria. For example, the largest known bacterial N-end rule, in the Gram-negative bacterium *Vibrio vulnificus*, is similar to the N-end rule of the eukaryotic Arg/N-end rule pathway³⁷ (Fig. 2A, Fig. 4B and Fig. 5). Remarkably, however, this similarity results from different mechanisms in bacteria versus eukaryotes: the N-terminal Arg and Lys are Nd^p (primary destabilizing) residues in the eukaryotic Arg/N-end rule pathway, in that these basic residues (and N-terminal His as well) are recognized directly by the UBR domains (type-1 binding sites) of E3 N-recognins such as Ubr1 (Fig. 2A, Fig. 3, C, Fig. 5 and Fig. 8A, B)⁸³⁻⁸⁵. In contrast, the N-terminal Arg and Lys are Nd^s (secondary destabilizing) residues in both *E. coli* and *V. vulnificus*, owing to the presence of the Aat L/F-transferase. This enzyme, which conjugates Leu (and occasionally Phe) to the N-terminal Arg or Lys residues^{14,71,316-318} (Fig. 5), is absent from examined eukaryotes. The Nt-leucylating bacterial Bpt L-transferases are sequelogous¹ to Nt-arginylating Ate1 R-transferases of eukaryotes (Fig. 6A and ref. 37). Strikingly, however, bacterial Bpt L-transferases are largely nonsequelogenous to bacterial Aat L/F-transferases³⁷, despite the similarity of reactions catalyzed by Bpt and Aat (Fig. 5).

One difference between bacterial and eukaryotic N-end rule pathways is particularly remarkable in its consistency (no exceptions so far) and the absence of a robust explanation for its emergence on either functional or mechanistic grounds. Specifically, the bacterial Leu/N-end rule pathway employs Nt-leucylation of N-end rule substrates, whereas the otherwise similar eukaryotic Arg/N-end rule pathway employs Nt-arginylation (Fig. 2A, Fig. 3 and Fig. 5). The leucylation/arginylation dichotomy exists despite a significant sequelogy¹ (sequence similarity) between the Nt-leucylating Bpt transferases of bacteria and the Nt-arginylating Ate1 R-transferases of eukaryotes (Fig. 6A and ref. 37). This sequelogy suggests homology, i.e., a common ancestry of Bpt and Ate1. Nevertheless, the Nt-arginylating and Nt-leucylating transferases are cleanly divided, in extant organisms, between Bpt L-transferases in bacteria and Ate1 R-transferases in eukaryotes. This dichotomy is even more remarkable than described above. Specifically, *Plasmodium falciparum*, an obligatory intracellular parasite and the cause of malaria in humans, is a eukaryote that contains a *Plasmodium*-specific R-transferase³⁷. However, this R-transferase, which is also present in other apicomplexans (a group of obligately parasitic unicellular eukaryotes that includes *Plasmodium*) but not in other examined eukaryotes, is a sequelog *not* of the bacterial Bpt L-transferase but of the bacterial Aat L/F-transferase³⁷. (This would not be expected *a priori*, given the sequelogy between bacterial Bpt and non-*Plasmodium* eukaryotic Ate1 R-transferases.)

Thus, irrespective of whether an Nt-arginylating R-transferase of a eukaryotic cell descended from a Bpt-type L-transferase or from a largely nonsequelogenous Aat-type L/F-transferase (Fig. 5), the final result was, in all cases, the change of enzymatic specificity from that of L-transferase (Nt-leucylation) to that of R-transferase (Nt-arginylation). Why? There is no clear answer (to the best of my knowledge), in part because the replacement of Nt-leucylation in bacteria with Nt-arginylation in eukaryotes does not appear to have been necessary for 'mechanistic' reasons. Specifically, most UBR-type N-recognins in the Arg/N-end rule pathway of eukaryotes contain a type-2 substrate-binding site whose ability to recognize the N-terminal Leu, Phe, Tyr Trp or Ile residues of N-end rule substrates is essentially identical to the recognition specificity of ClpS, the bacterial N-recognin (Fig. 5, Fig. 6B-D and Fig. 7A). Thus the presumably ancestral Nt-leucylation should have sufficed in eukaryotes, without a change to Nt-arginylation. However, Nt-arginylation did emerge in eukaryotes and replaced Nt-leucylation both completely and early in eukaryotic evolution. Specifically, the *S. cerevisiae* and human Ate1 R-transferases are highly sequelogenous enzymes. In

addition, there is no Nt-leucylation in examined eukaryotes and there is no Nt-arginylation in examined prokaryotes.

For reasons suggested by Lynch²¹⁰ (Section 6), many aspects of molecular circuits in eukaryotic cells evolved through a genetic drift and occasional fixation of mildly deleterious mutations, as distinguished from adaptive Darwinian evolution based on positive selection. Although an initially nonadaptive evolution may be relevant to the emergence of Nt-arginylation in eukaryotes, it is most likely that a specific selection pressure favored, in primordial eukaryotes, a replacement of the (apparently) preceding Nt-leucylation by Nt-arginylation. What might be a reason for such a pressure? Discussions of LUCA and early evolution suffer from the problem that a less parsimonious, more convoluted scenario would be unlikely *a priori* (because of Occam's razor) but cannot be formally precluded. With this caveat, one difference between Arg and Leu that may have caused the ascendancy of Nt-arginylation in eukaryotes is high reactivity of the side chain of Arg, in comparison to Leu, and a multitude of metabolic transitions that involve Arg, often in the context of the Arg/N-end rule pathway. For example, Arg is a substrate of NO synthases and the immediate precursor of NO. Furthermore, NO is required for the *in vivo* oxidation of N-terminal Cys, a modification that allows Nt-arginylation of the resulting Cys-sulfinate or Cys-sulfonate by the Ate1 R-transferase of the Arg/N-end rule pathway³² (Fig. 3). Arg-tRNA is a cosubstrate of R-transferase and at the same time the source of Arg in proteins produced by the ribosomes, suggesting a competition between these uses of Arg. (But an analogous competition also exists for the consumption of Leu-tRNA by L-transferases versus bacterial ribosomes.) Some Arg residues in proteins undergo enzymatic methylation or deimination, the latter a conversion of the positively charged Arg to the uncharged citrulline residue^{338,339}.

Might the *in vivo* methylation or deimination of Arg in cellular proteins involve the N-terminal Arg residue as well? Might there be a regulatory connection, through Arg-tRNA and R-transferase, between the Arg/N-end rule pathway and translation by ribosomes? (Interestingly, the mouse Ate1 R-transferase binds to specific mRNAs and is associated with translationally active polysomes (R.-G. Hu, H. Wang and A.V., unpubl. data; see also ref. 114.) These are some among a multitude of questions that can be asked about the Arg/N-end rule pathway. The answers at hand have revealed a strikingly multifunctional, universally present and extensively regulated proteolytic circuit (Fig. 2A and Fig. 3). Explorations of bacterial and mitochondrial N-end rule pathways (Sections 10 and 11) continue as well. The recent discovery of the Ac/N-end rule pathway (Fig. 2B

and Fig. 4A) further expanded the already broad functional scope of the eukaryotic N-end rule pathway, and has also revealed the main physiological functions of Nt-acetylases and Met-aminopeptidases³ (Sections 3 and 8). Many years after the initial discovery of the N-end rule⁴, this ancient system continues to be a fount of biological insights.

Acknowledgments

I thank T. Baker, D. Barford, C. Brower, D. Finley, M. Lynch, A. Shemorry, and B. Wadas for their helpful comments on the manuscript. I am particularly grateful to D. Finley for his detailed suggestions. I also thank D. Barford, H. K. Song, and K. Zeth for their assistance with and advice about figures. Studies in our laboratory are supported by grants from the National Institutes of Health and the March of Dimes Foundation.

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Figure Legends

Figure 1. The ubiquitin-proteasome system, the ubiquitin fusion technique, and N-terminal processing of newly formed proteins. **A:** The ubiquitin-proteasome system (Ub system)^{6,45-65}. The conjugation of Ub to other proteins involves a preliminary ATP-dependent step in which the last residue of Ub (Gly⁷⁶) is joined, via a thioester bond, to a Cys residue of the E1 (Ub-activating) enzyme. The ‘activated’ Ub moiety is transferred to a Cys residue in one of several Ub-conjugating (E2) enzymes, and from there, through an isopeptide bond, to a Lys residue of an ultimate acceptor, denoted as ‘protein’. E2 enzymes function as subunits of E2-E3 Ub ligase complexes that can produce substrate-linked poly-Ub chains. Such chains have specific Ub-Ub topologies, depending on the identity of a Lys residue of Ub (which contains several lysines) that forms an isopeptide bond with C-terminal Gly⁷⁶ of the adjacent Ub moiety in a chain. Specific poly-Ub chains can confer either the degradation of a substrate by the 26S proteasome or other metabolic fates. Monoubiquitylation of some protein substrates can also occur, and has specific functions. One role of E3 is the initial recognition of a substrate’s degradation signal (degron). Individual mammalian genomes encode at least a 1,000 distinct E3 Ub ligases. **B:** The Ub fusion technique^{4,213}. In eukaryotes, linear fusions of Ub to other proteins are cotranslationally cleaved by deubiquitylases at the last residue of Ub, making it possible to produce, *in vivo*, different residues at the N-termini of otherwise identical proteins. **C:** N-terminal processing of nascent proteins by N^α-terminal acetylases (Nt-acetylases) and Met-aminopeptidases (MetAPs). ‘Ac’ denotes the N^α-terminal acetyl moiety. M, Met. X and Z, single-letter abbreviations for any amino acid residue. Yellow ovals denote the rest of a protein. **D:** Met-aminopeptidases cleave off the N-terminal Met residue if a residue at position 2 belongs to the set of residues shown¹⁰¹. Gly and Pro at position 2 are depicted in a different color because these residues, in contrast to other small residues, are rarely Nt-acetylated after the removal of N-terminal Met (Fig. 2B).

Figure 2. The N-end rule pathway in *Saccharomyces cerevisiae*. **A:** The Arg/N-end rule pathway⁴⁴. See the main text for details. Yellow ovals denote the rest of a protein substrate. ‘Primary’, ‘secondary’ and ‘tertiary’ denote mechanistically distinct subsets of destabilizing N-terminal residues. The physically associated Ubr1 (N-recognin) and Ufd4 E3s have substrate-binding sites that recognize internal (non-N-terminal) degrons in substrates of the

Arg/N-end rule pathway that lack N-degrons. Ubr1 (but not Ufd4) recognizes N-degrons as well⁴⁴. **B:** The Ac/N-end rule pathway³. Red arrow on the left indicates the removal of N-terminal Met by Met-aminopeptidases. This Met residue is retained if a residue at position 2 is nonpermissive (too large) for Met-aminopeptidases (Fig. 1D). If the (retained) N-terminal Met or N-terminal Ala, Val, Ser, Thr and Cys are followed by residues that allow Nt-acetylation (see the main text), these N-terminal residues are usually Nt-acetylated⁹¹⁻⁹³. The resulting N-degrons are called ^{Ac}N-degrons. The term 'secondary' refers to the necessity of modification (Nt-acetylation) of a destabilizing N-terminal residue before a protein can be recognized by a cognate Ub ligase. Proteins containing ^{Ac}N-degrons are targeted for ubiquitylation and proteasome-mediated degradation by the Doa10 E3 N-recognin, in conjunction with the Ubc6 and Ubc7 E2 enzymes⁴⁴. Although Gly and Pro can be made N-terminal by MetAPs, and although Doa10 can recognize Nt-acetylated Gly and Pro, few proteins with N-terminal Gly or Pro are Nt-acetylated⁹¹⁻⁹³.

Figure 3. The mammalian Arg/N-end rule pathway. See the main text for details. N-terminal residues are indicated by single-letter abbreviations for amino acids. Yellow ovals denote the rest of a protein substrate. 'Primary', 'secondary' and 'tertiary' denote mechanistically distinct subsets of destabilizing N-terminal residues. C* denotes oxidized N-terminal Cys, either Cys-sulfinate or Cys-sulfonate, produced *in vivo* through reactions that require both nitric oxide (NO) and oxygen^{32,33}. The mammalian N-recognins Ubr1, Ubr2, Ubr4 and Ubr5 (Edd) have multiple substrate binding sites that also recognize internal (non-N-terminal) degrons in other substrates of the Arg/N-end rule pathway, the ones that lack N-degrons. A question mark after Trip12 (which mediates the mammalian UFD pathway²⁵⁸ and is a sequelog of the *S. cerevisiae* Ufd4 E3) denotes the untested possibility that mammalian Ubr1 and/or Ubr2 form complexes with Trip12, by analogy with the Ubr1-Ufd4 complex in *S. cerevisiae* (Fig. 2A).

Figure 4. Rule books of N-end rules. **A:** The N-end rule in eukaryotes. It results from combined activities of the Arg/N-end rule and Ac/N-end rule pathways³. In eukaryotes that produce NO, the N-terminal Cys residue (in yellow rectangles) can be targeted, alternatively, by either one of the two branches of the N-end rule pathway, with oxidized Cys marked by an asterisk (see Section 3). **B:** A comparison of rule books of N-end rule pathways in different organisms, indicated on the left. Black circles, blue or green triangles, and red crosses denote primary (Nd^P), secondary (Nd^S) and

tertiary (Nd^t) destabilizing N-terminal residues, respectively. Blue triangles denote secondary destabilizing N-terminal residues that involve either Nt-leucylation (in bacteria) or Nt-arginylation (in eukaryotes). Green triangles denote secondary destabilizing N-terminal residues that involve Nt-acetylation³. N-terminal Cys is denoted by both a green triangle and a red cross, given its alternative functioning as a part of NO/O₂-mediated N-degrons or ^{Ac}N-degrons. Open circles, in bacterial N-end rules, denote stabilizing ('non-destabilizing') N-terminal residues. Yellow circles, in eukaryotic N-end rules, denote Pro and Gly. These N-terminal residues are rarely Nt-acetylated and therefore, operationally, are usually stabilizing ('non-destabilizing') residues. But in some proteins with N-terminal Pro or Gly these residues can be Nt-acetylated. If other components of an ^{Ac}N-degron are also in place (see Section 8), such proteins can become substrates of the Ac/N-end rule pathway³.

Figure 5. Bacterial N-end rule pathways. **A:** The *E. coli* Leu/N-end rule pathway^{14,16,37}. See Sections 3, 6 and 10 for details. The Aat L/F-transferase conjugates (largely) Leu to N-terminal Arg or Lys. N-end rule substrates bearing primary (bulky hydrophobic) destabilizing N-terminal residues are recognized by the ClpS N-recognin and are delivered for degradation to the ClpAP protease. **B:** The Leu/N-end rule pathway in another gram-negative bacterium, *V. vulnificus*, which contains both the Aat L/F-transferase and the Bpt L-transferase. As a result, N-terminal Asp and Glu, which are stabilizing ('non-destabilizing') residues in *E. coli*, are secondary destabilizing residues in the *V. vulnificus* Leu/N-end rule pathway³⁷.

Figure 6. Bpt L-transferases and ClpS N-recognins. **A:** Sequence alignments of bacterial Bpt L-transferases and eukaryotic Ate1 R-transferases³⁷. The sequelogy (sequence similarity¹) between Bpt and Ate1 encompasses more of their sequences than shown here³⁷. Ate1 R-transferases lack a sequence motif (its consensus, in red, is shown at the top of the diagram) that Bpt L-transferases uniformly contain. This motif is characteristic of proteins that bind to a Fe-S cluster^{319,320} (Section 10). **B:** Surface representation of the C-terminal domain of the *E. coli* ClpS N-recognin in a complex with an 11-mer peptide (shown as a stick model) that bears N-terminal Leu, a primary destabilizing (Nd^p) residue in the Leu/N-end rule pathway⁷⁴. Blue sphere, water molecule. **C:** Ribbon representation of the full-length 12-kDa ClpS in the same complex. **D:** Sequence alignments of the ~70-residue domain of bacterial ClpS N-recognins in *Caulobacter crescentus*, *E. coli*, *Deinococcus*

radiodurans, *Helicobacter pylori*, *Synechocystis* sp. PCC6803 (the latter a photosynthesis-capable cyanobacterium), and in chloroplast (*A. thaliana*). This region of ClpS binds to N-terminal Nd^p residues of the Leu/N-rule pathway. ClpS sequences are aligned with sequelogous regions of eukaryotic (*S. cerevisiae*, *D. melanogaster*, *M. musculus*, *H. sapiens*) Ubr1 N-recognins (they are ~20-fold larger than ClpS) that encompass the type-2 substrate-binding site of Ubr1 (Fig. 7A). The specificity of this (type-2) Ubr1 binding site for bulky hydrophobic N-terminal Nd^p residues is nearly the same as the specificity of ClpS, except that the Ubr1 site binds to N-terminal Ile as well (Fig. 2A), in contrast to ClpS (see the main text). Arrowheads indicate the positions of crystallographically determined contacts between the ClpS of *C. crescentus* and an N-end rule peptide⁷³. Black cylinders indicate α -helices in this region of ClpS.

Figure 7. Structural organization and phosphorylation of the Ubr1 N-recognin.

A: Phosphorylated residues of the *S. cerevisiae* Ubr1 E3 N-recognin of the Arg/N-end rule pathway are indicated above the diagram³⁹. The regions containing the type-1 substrate-binding site (UBR domain), the type-2 substrate-binding site, the BRR (basic residues-rich) domain, the Cys/His-rich RING domain and the AI (autoinhibitory) domain^{15,22,29,39,83-85} are also indicated. **B:** The ‘primed’ cascade of Ubr1 phosphorylation³⁹. The initial phosphorylation of Ubr1 on Ser³⁰⁰ by the Yck1/Yck2 kinases of the casein kinase type-I family makes possible (‘primes’) the subsequent, apparently sequential phosphorylation of Ubr1 by Mck1, a Gsk3-type kinase, on Ser²⁹⁶, Ser²⁹², Thr²⁸⁸ and Tyr²⁷⁷. Also indicated is the identified function of the Ser³⁰⁰ phosphorylation of Ubr1 in the control of peptide import³⁹.

Figure 8. UBR domains in N-recognins and putative N-recognins of the mammalian Arg/N-end rule pathway. **A:** Ribbon diagram of the ~80-residue *S. cerevisiae* UBR domain⁸³ (Fig. 7A) in the complex with the RLGES peptide that bears N-terminal Arg, a type-1 Nd^p residue²⁸. The bound RLGES is shown as a stick model, with carbon atoms colored yellow. Several residues are marked with a black sphere and numbered to facilitate the tracing of the polypeptide chain. The names of residues of the RLGES peptide are in red, with the letter ‘s’ (‘substrate’) appended to their position numbers. Side chains of residues in the UBR domain that are present near missense mutations in UBR1 of patients with Johanson-Blizzard syndrome (JBS) (C.-S. Hwang, M. Zenker, H. K. Song and A.V., unpubl. data) are shown in a stick form, with carbon atoms colored green.

Three coordinated zinc ions of the UBR domain⁸³ are shown as red spheres. **B:** Molecular surface of the *S. cerevisiae* UBR domain. Negatively and positively charged surfaces are shaded red and blue, respectively. The bound RLGES peptide is shown in yellow. Some residues of Ubr1 that comprise the N-degron-binding cleft are labeled⁸³. **C:** Diagram of the mammalian UBR-domain family of E3 Ub ligases, showing both UBR and other domains of these E3s (RING, HECT, PHD, CRD and F-box) that contribute to recognition and ubiquitylation of protein substrates^{31,43}. Ubr1, Ubr2, Ubr4, and Ubr5/Edd of this set are operationally defined N-recognins of the mammalian Arg/N-end rule pathway in that they specifically bind to the type-1 and/or type-2 destabilizing N-terminal residues, whereas Ubr3, Ubr6, and Ubr7 are not N-recognins^{43,274} (Section 9.1).

Figure 9. Splicing-derived isoforms of the *Ate1*-encoded Arg-tRNA-protein transferase (R-transferase) and its inhibition by hemin. **A:** The bidirectional *DfaP_{Ate1}* promoter (containing a CpG island) upstream of exon 1B of the mouse *Ate1* gene^{35,282}. Green arrows indicate transcriptional units oriented in both directions from *DfaP_{Ate1}*, and also from an unmapped ‘upstream’ promoter that mediates the expression of *Ate1* transcripts containing exon 1A. The locations and sizes of some *Ate1* exons are shown as well. **B:** The exons, including alternative exons, of the mouse *Ate1* gene, with deduced lengths of the corresponding polypeptide segments indicated on top. **C:** Mouse R-transferase isoforms (and their designations) that are produced through alternative splicing of *Ate1* pre-mRNA. **D:** Sequence comparisons of translated vertebrate *Ate1* exons 1A amongst themselves and with the set of longer but also sequelogous alternative exons 1B. Most of recurrent amino acid identities are highlighted by color. *Mus musc.*, mouse; *Rattus norv.*, rat; *Homo sap.*, human; *Gallus gall.*, chicken. **E:** The mouse ATE1^{1B7A} isoform, with locations of significant Cys-containing motifs, including the vicinal Cys⁷¹ and Cys⁷² residues. A disulfide bond between them is the result of hemin-mediated oxidation and functional inactivation of R-transferase³⁵. **F:** Diagram of the previously proposed³⁵ redox mechanism of the hemin-mediated disulfide formation between Cys⁷¹ and Cys⁷² of Ate1.

Figure 10. Confirmed and putative N-end rule substrates produced by caspases and other nonprocessive proteases. Amino acid residues are indicated by single-letter abbreviations. Arrowheads and enlarged residues, in red, indicate the cleavage sites and N-terminal residues of the corresponding C-terminal fragments. A number on the left represents the first residue of a protein (numbered as in the full-length protein) that is shown in the diagram. A number on the right

represents the last residue of a full-length protein. The prefixes *Dm*, *Hs*, *Mm*, and *Sc* refer to proteins of *D. melanogaster*, *H. sapiens*, *M. musculus* and *S. cerevisiae*, respectively. See Section 5 for a description of specific protein fragments cited in this list.

Figure 11. Organization and *cis-trans* targeting of eukaryotic N-degrons. **A:** Three determinants of N-degron. *d*, a destabilizing N-terminal residue. *K*, a ‘ubiquitylatable’ internal Lys residue. The absence of one of these determinants abrogates polyubiquitylation of a protein, despite the presence of another determinant. The third determinant of N-degron is an unstructured region that is required for polyubiquitylation and/or the initiation of degradation of a polyubiquitylated N-end rule substrate by the 26S proteasome. See Section 5 for references and details. **B** and **C:** *cis* versus *trans* polyubiquitylation of an oligomeric N-end rule substrate that results in the degradation of a subunit that becomes linked to a poly-Ub chain¹³ (see Section 5). **D:** *trans*-degradation, in which a specific subunit of oligomeric protein is polyubiquitylated but is not degraded by the 26 proteasome, for example, because it lacks an unstructured region that is required for the initiation of degradation. Instead, a subunit-selective degradation of another, non-ubiquitylated subunit takes place. This mode of degradation was demonstrated by the Matouschek laboratory¹⁶⁸ for oligomeric substrates of the UFD pathway. It remains to be determined whether the analogous (hypothetical) *trans*-degradation of an oligomeric N-end rule substrate can also occur. **E:** The 1989–1996 hairpin insertion model of protein targeting by the 26S proteasome⁵. No details of the 26S proteasome structure (such as the 19S regulatory particle (RP)) are shown in this 1996 diagram⁵, and the sizes of specific components such as Ub moieties, the poly-Ub chain and the proteasome, are not to scale (see Section 5).

Figure 12. Targeting and degradation of N-end rule substrates by the bacterial Leu/N-end rule pathway. This model, proposed by Román-Hernandez *et al.*⁸² and based on studies by the Baker, Sauer, Bukau, Maurizi and other laboratories^{16,75,76,78,81,82}, is described in the main text. A folded polypeptide chain of an N-end rule substrate (in purple color) is depicted ‘explicitly’, in contrast to solid-body renderings of ClpS and ClpAP. Black circles in the ClpP moiety indicate its proteolytic active sites. See Section 5 for details.

Figure 13. Steric shielding of the Nt-acetylated N-terminal residue of a subunit in a protein complex. Shown here is a part of the crystal structure, by the Barford laboratory, of a complex

between the Hcn1 and Cut9 subunits of the *S. pombe* APC/C Ub ligase²⁴⁷. In this structure, the Nt-acetylated N-terminal Met residue of Hcn1 is enclosed within a chamber formed by the Cut9 subunit, including its interface with the other Cut9 subunit in the heterotetramer of Hcn1 and Cut9. N-terminal region of Hcn1 is shown in cyan as a stick model, and Cut9 is depicted as a cut-out surface representation, to show the chamber's interior²⁴⁷.

Figure 14. Spalogy (spatial similarity¹) between the Ntaq1 Nt^Q-amidase and Factor XIII transglutaminase. **A** and **B**: Crystal structures of the human Ntaq1 (C8orf32) Nt^Q-amidase³⁴⁰ (PDB 3C9Q) and FXIII transglutaminase³⁴¹ (PDB 1FIE), respectively. C8orf32 is an initially uncharacterized human protein the structure of which was deposited in the Protein Data Bank (PDB 3C9Q) by the Center for Eukaryotic Structural Genomics³⁴⁰, and was later shown, by Wang et al.⁴², to be the Ntaq1 Nt^Q-amidase. **C** and **D**: Structures around the active sites of Ntaq1 Nt^Q-amidase and Factor XIII transglutaminase, respectively. These regions are circled in **A** and **B**. **C** and **D**: The catalytic triad (Cys³¹⁴, His³⁷³, and Asp³⁹⁶) of Factor XIII transglutaminase (**D**) (ref. 341) and the corresponding residues (Cys²⁸, His⁸¹, and Asp⁹⁷) of human Ntaq1 (**C**) are indicated. Despite the striking spalogy between these regions of two enzymes (**C** and **D**), there is no significant sequelogy (sequence similarity) between them⁴².

Figure 15. Regulation of peptide import by the Arg/N-end rule pathway in *S. cerevisiae*, and inputs by the amino acid-sensing SPS pathway. **A**: The 'primed' cascade of Ubr1 phosphorylation in which the Yck1/Yck2-mediated phosphorylation on Ser³⁰⁰ of Ubr1 is essential for the normal regulation of peptide import³⁹ (see also the legend to Fig. 7A, B). **B**: Ubr1-mediated regulation of peptide import, and the involvement of the SPS pathway^{27,29,138,295,296}. Cup9 is a transcriptional repressor of the regulon that includes *PTR2*, which encodes the major importer of di/tripeptides. In the absence of Ubr1 (in *ubr1Δ* cells), Cup9 becomes long-lived, accumulates to high levels, and extinguishes expression of *Ptr2*. Therefore *ubr1Δ* cells cannot import di/tripeptides. In wild-type (*UBR1*) cells growing in the absence of extracellular di/tripeptides, a relatively low but non-zero number of Ubr1 molecules have their third substrate-binding site 'open' (not autoinhibited) and therefore can target Cup9 for degradation ($t_{1/2} \sim 5$ min) via its internal degron, resulting in a low but significant steady-state concentration of Cup9 and thus a weak but significant expression of the *Ptr2* transporter. In wild-type (*UBR1*) cells growing in the presence of extracellular di/tripeptides (some of which bear

type-1 and type-2 destabilizing N-terminal residues), the imported peptides interact with the type-1 and type-2 binding sites of Ubr1. This binding allosterically increases the fraction of Ubr1 molecules whose third (Cup9-specific) site is 'open' (active). The resulting decrease in the half-life of Cup9 (from ~5 min to below 1 min) results in a low concentration of Cup9, and consequently to a strong induction of the Ptr2 transporter^{27,29,138}. Also shown is the amino acid-sensing SPS pathway (see Section 9.4 for details and additional references), which can influence the import of peptides at least in part through the Yck1/Yck2-mediated phosphorylation of Ubr1 on Ser³⁰⁰. This phosphorylation is required (through a mechanism that remains to be determined) for normal levels of Ubr1 activity in the Ptr2-Cup9-Ubr1 circuit³⁹.

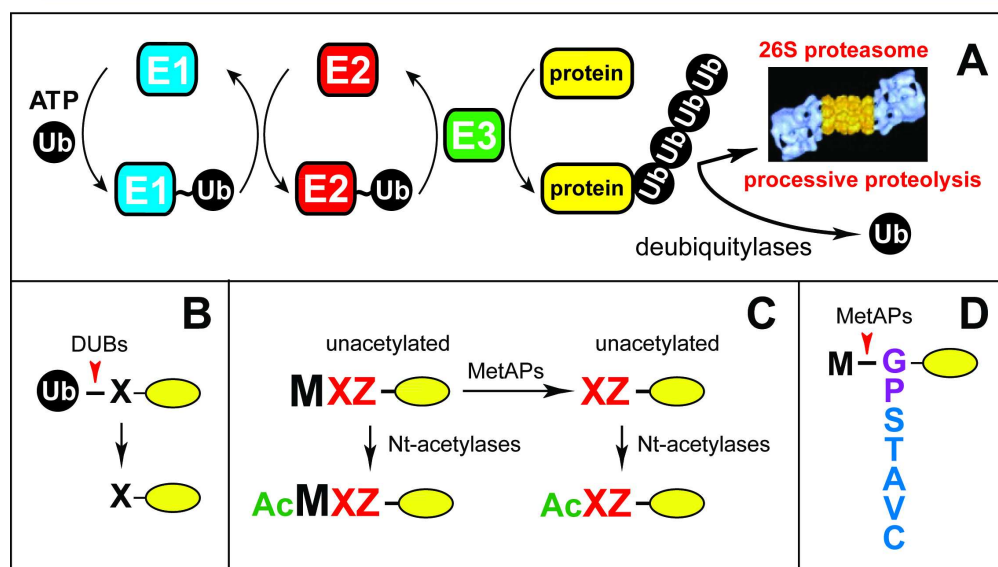


Fig. 1, Varshavsky

Fig 1-Varshavsky-PRO-11-0142
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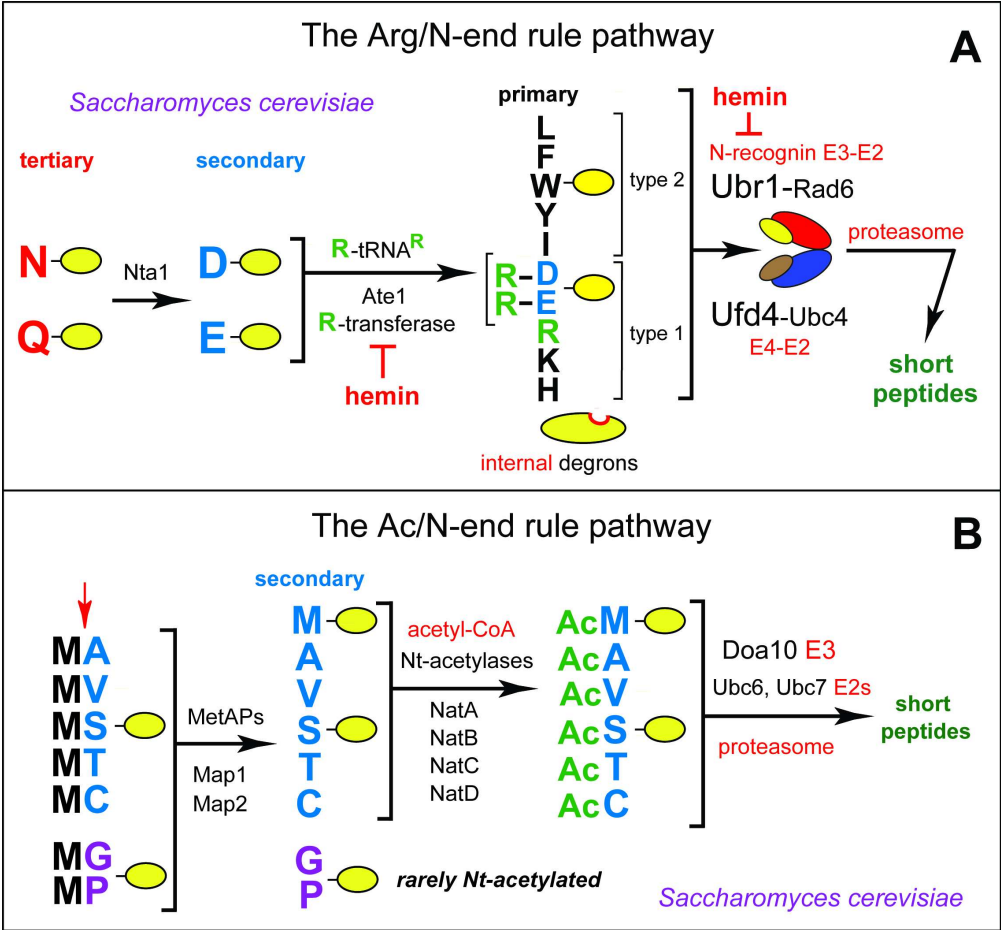


Fig. 2, Varshavsky

Fig 2-Varshavsky-PRO-11-0142
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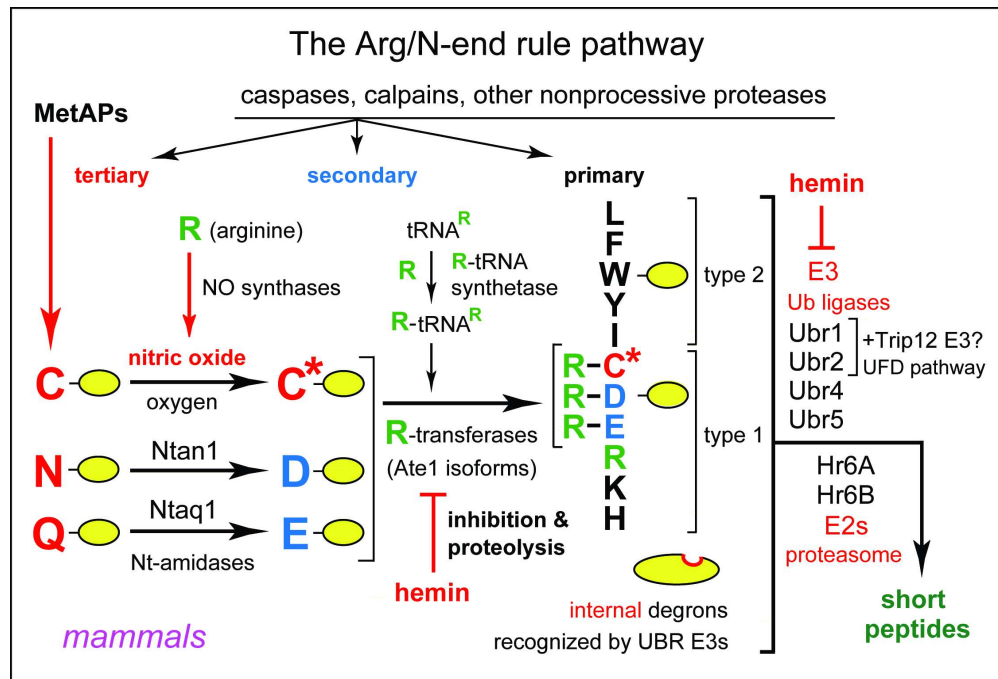


Fig. 3, Varshavsky

Fig 3-Varshavsky-PRO-11-0142
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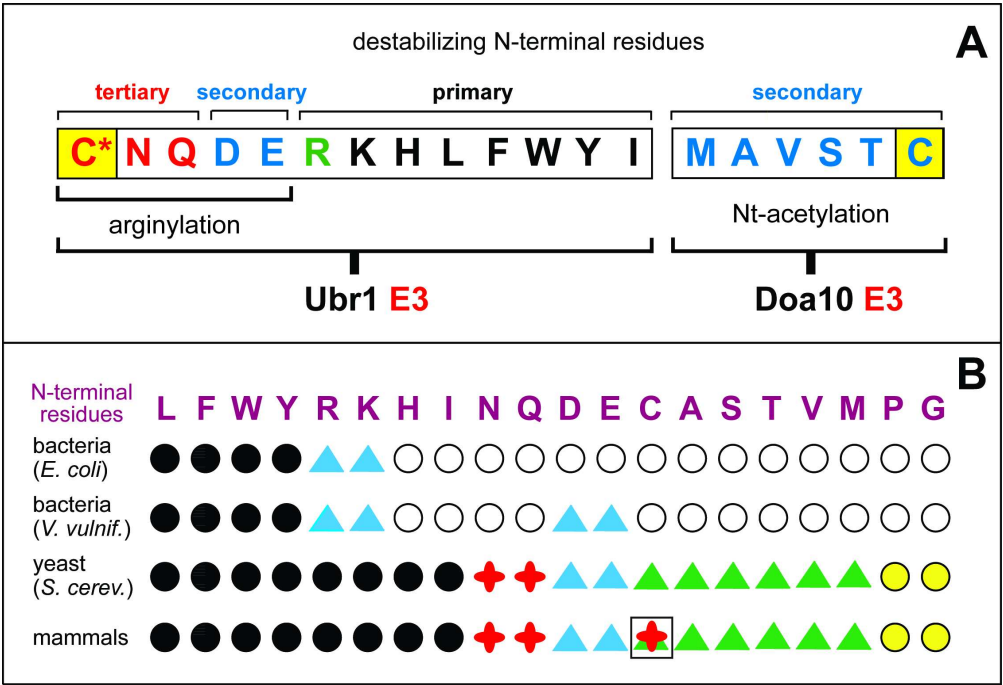


Fig. 4, Varshavsky

Fig 4_Varshavsky_Pro_11-0142
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The Leu/N-end rule pathway

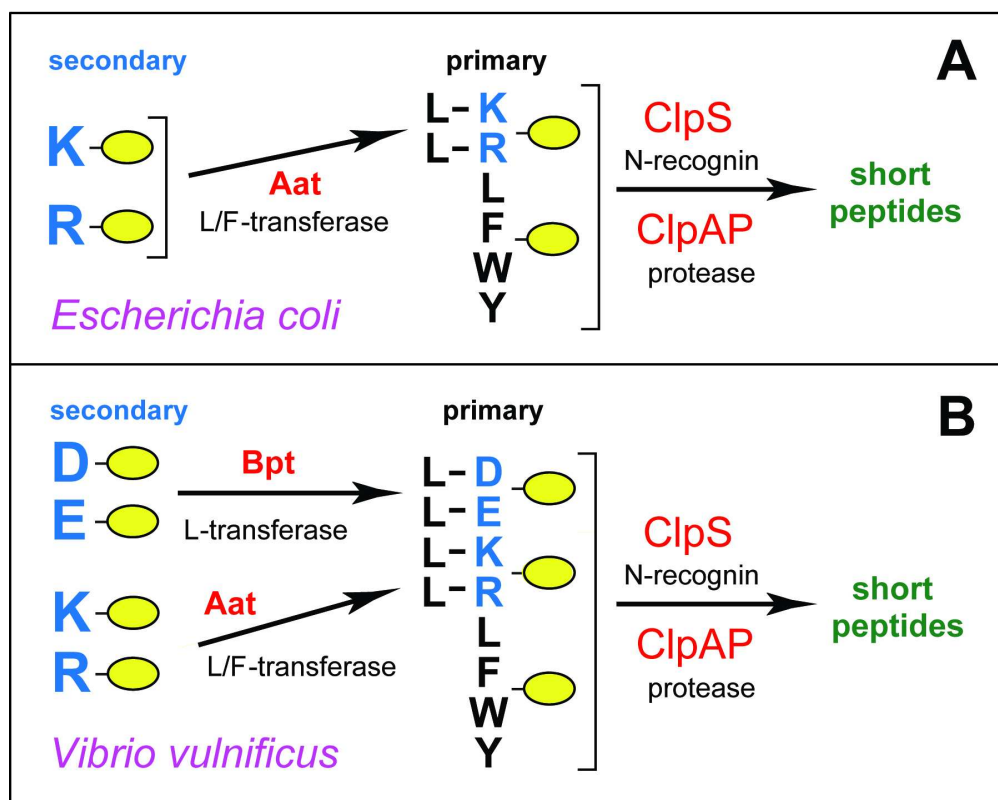


Fig. 5, Varshavsky

Fig 5_Varshavsky_Pro_11-0142
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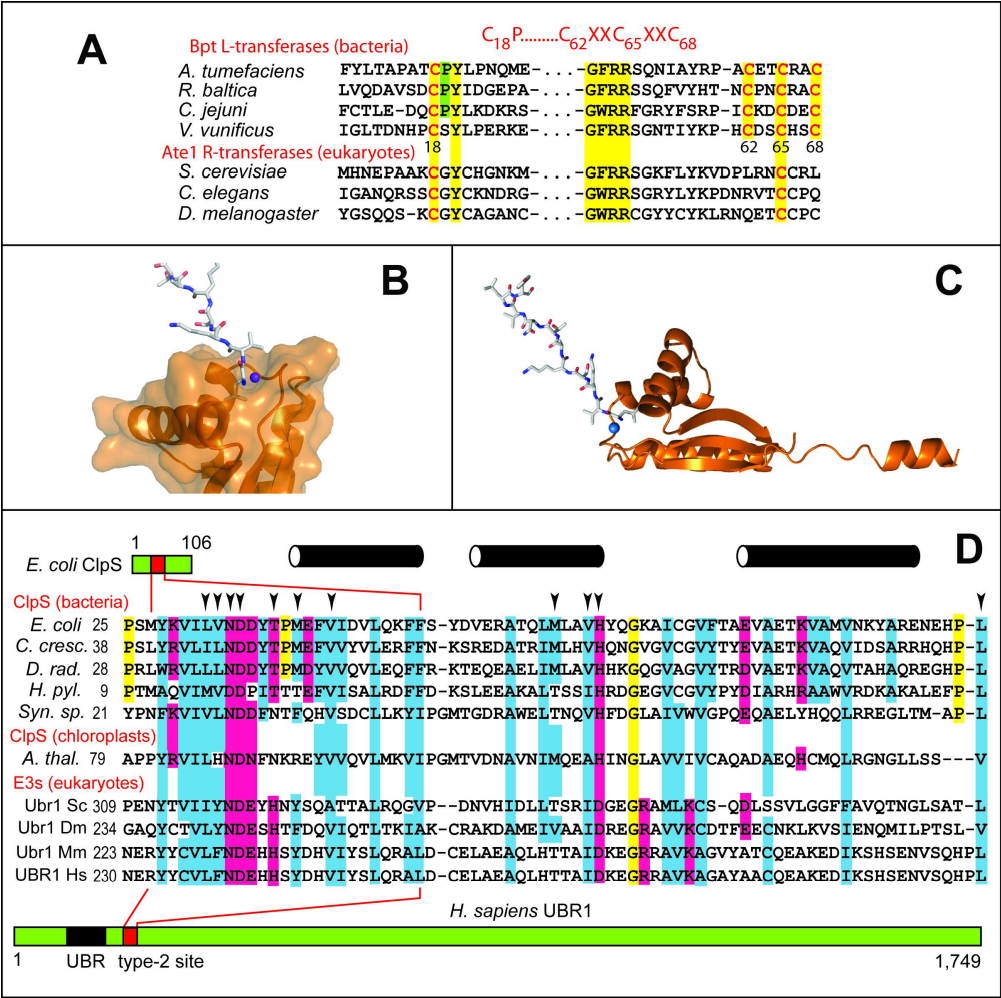


Fig. 6, Varshavsky

Fig 6_Varshavsky_Pro_11-0142
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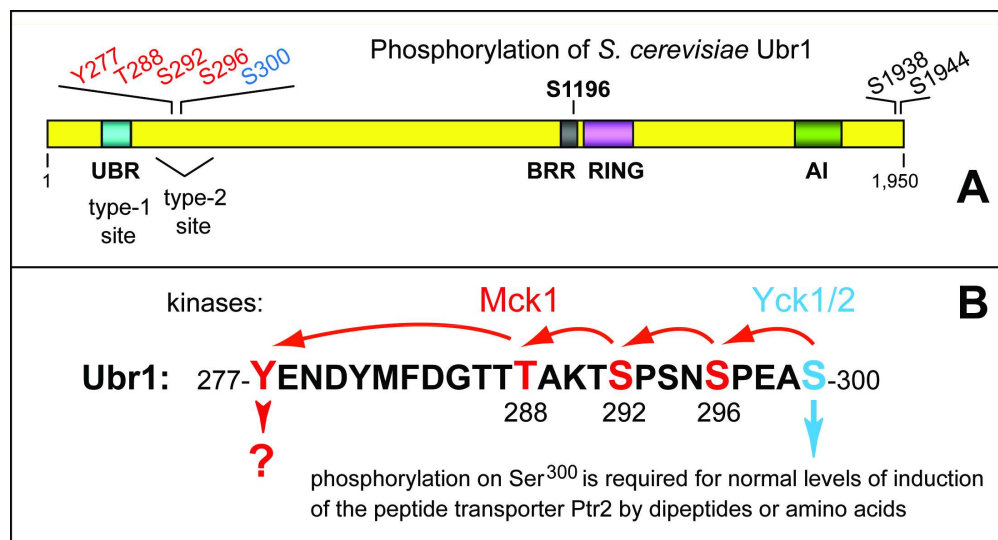


Fig. 7, Varshavsky

Fig 7_Varshavsky_PRO_11-0142
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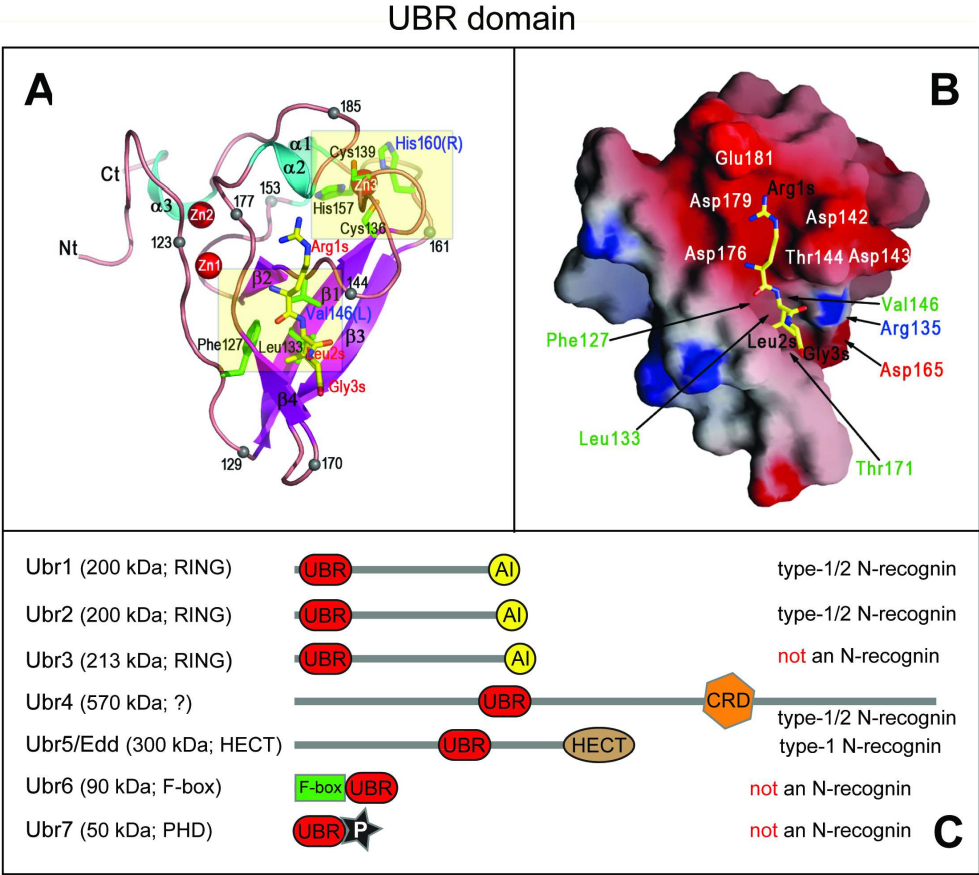


Fig. 8, Varshavsky

Fig 8_Varshavsky_PRO_11-0142
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Ate1 arginyl-tRNA-protein transferase (R-transferase)

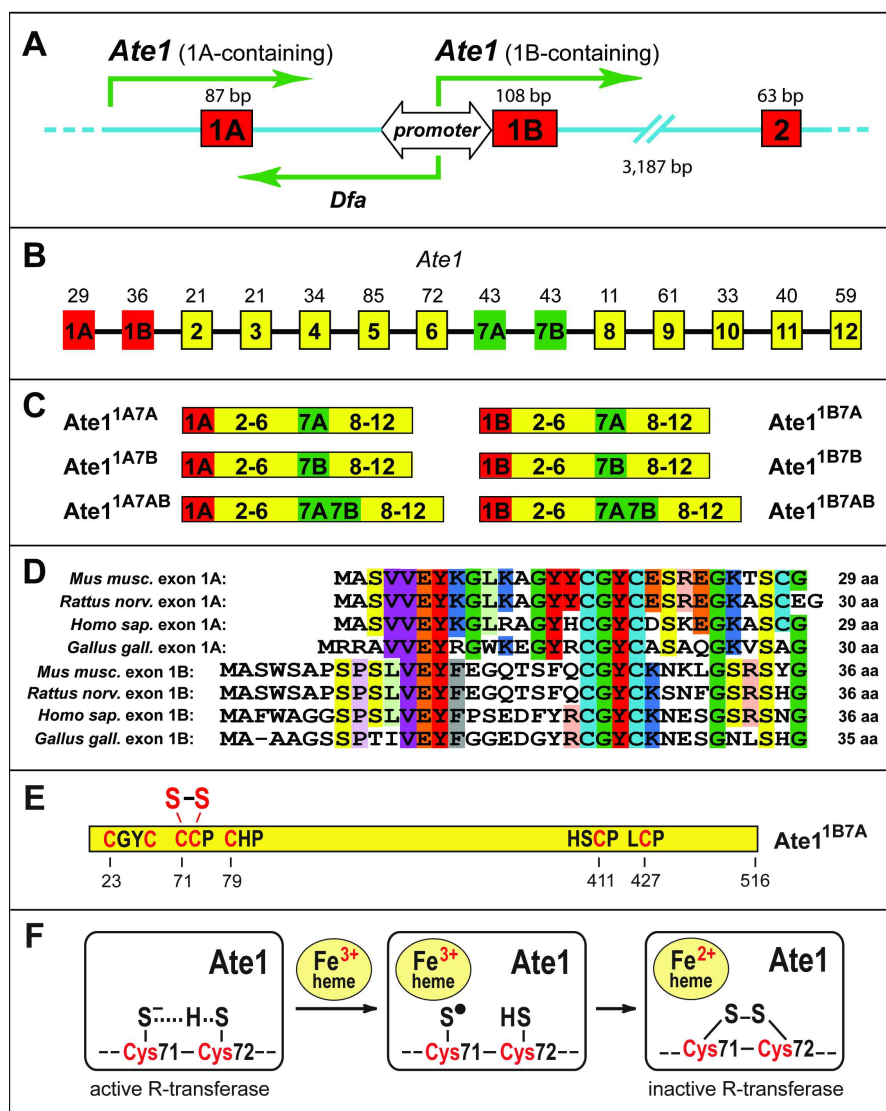


Fig. 9, Varshavsky

Fig 9_Varshavsky_PRO_11-0142
193x260mm (300 x 300 DPI)

Protein	Cleavage site	N-terminal residue	Fate and/or activity of a C-terminal fragment
Identified substrates of the Arg/N-end rule pathway			
Dm-DIAP1	16 FDQVD ^Y NNTN....S 438	Nd ^t	Caspase-processed antiapoptotic DIAP1 is a short-lived N-end rule substrate, degraded via Nt-deamidation and Nt-arginylation.
Hs-RGS4	1 M ^Y C ^K GLAGL....A 205	Nd ^t	Cotranslational removal of N-terminal Met by Met-aminopeptidases creates N-degrons of these regulators of G-proteins, thereby making RGS4, RGS5 and RGS16 conditionally short-lived proteins. The activity of their N-degrons is controlled by nitric oxide (NO) and O ₂ .
Hs-RGS5	1 M ^Y C ^K GLAAL....A 181		
Hs-RGS16	1 M ^Y C ^R TLAAF....A 202		
Hs-RAD21	167 DREIMR ^Y EGSA....I 631	Nd ^S	A fragment of the Scc1/Rad21 cohesin subunit in yeast and mammals is produced by separase. High fidelity of chromosome segregation requires degradation of this fragment by the Arg/N-end rule pathway.
Sc-Scc1	175 SLEVGR ^Y RFSP....A 566	Nd ^P	
HIV integrase	GIRKVL ^Y FDG....D 288	Nd ^P	HIV integrase is produced through processing of the precursor polyprotein and is a short-lived N-end rule substrate in infected cells.
Lm-LLO	19 AQQTEA ^Y KDAS....E 529	Nd ^P	Lysteriolysin O (LLO), a toxin of the bacterium <i>L. monocytogenes</i> , is a short-lived N-end rule substrate in the cytosol of mammalian cells.
Some of putative substrates of the Arg/N-end rule pathway			
Hs-HEF1	626 MDDYD ^Y VHL....F 834	Nd ^P	The C-terminal fragment of the focal adhesion mediator HEF1 is produced by caspases during mitosis, and causes rounding of cells.
Hs-MET	998 NESVD ^Y RAT....S 1390	Nd ^P	C-terminal proapoptotic fragment of the receptor MET is produced by caspases when MET is not associated with its cognate ligands.
Hs-MYC	293 SPLVLK ^Y RCHV....A 439	Nd ^P	This short-lived fragment of Myc is produced by calpain-mediated cleavage and has activities that differ from those of full-length Myc.
Hs-ETK	236 REDFPD ^Y WWQV....H 675	Nd ^P	This C-terminal fragment of the antiapoptotic Etk/Bmx Tyr kinase is produced by caspases and is proapoptotic.
Hs-EPHA4	768 SRVLED ^Y DPEA....V 986	Nd ^S	This C-terminal proapoptotic fragment of the EphA4 receptor is produced by caspases in the absence of EphB3, the ligand of EphA4.
Mm-Cdc42	116 QIDLRD ^Y DPST....F 191	Nd ^S	This C-terminal fragment of Cdc42, a Ras-like regulator GTPase, is produced by caspases.
Hs-MDM2	356 GFDVPD ^Y CKKT....P 491	Nd ^t	The Mdm2 E3 ubiquitin ligase, a regulator of p53, is cleaved by caspases, resulting in a metabolic stabilization of p53.
Hs-PKCδ	324 GEDMQD ^Y NSGT....D 676	Nd ^t	This caspase-produced, catalytically active C-terminal fragment of the protein kinase Cδ is proapoptotic, unlike the uncleaved PKCδ.
Hs-PKCθ	324 PLDEV ^Y KMCH....S 706	Nd ^P	This caspase-produced, catalytically active C-terminal fragment of the protein kinase Cθ is proapoptotic, unlike the uncleaved PKCθ.

Fig. 10, Varshavsky

Fig 10_Varshavsky_PRO_11-0142
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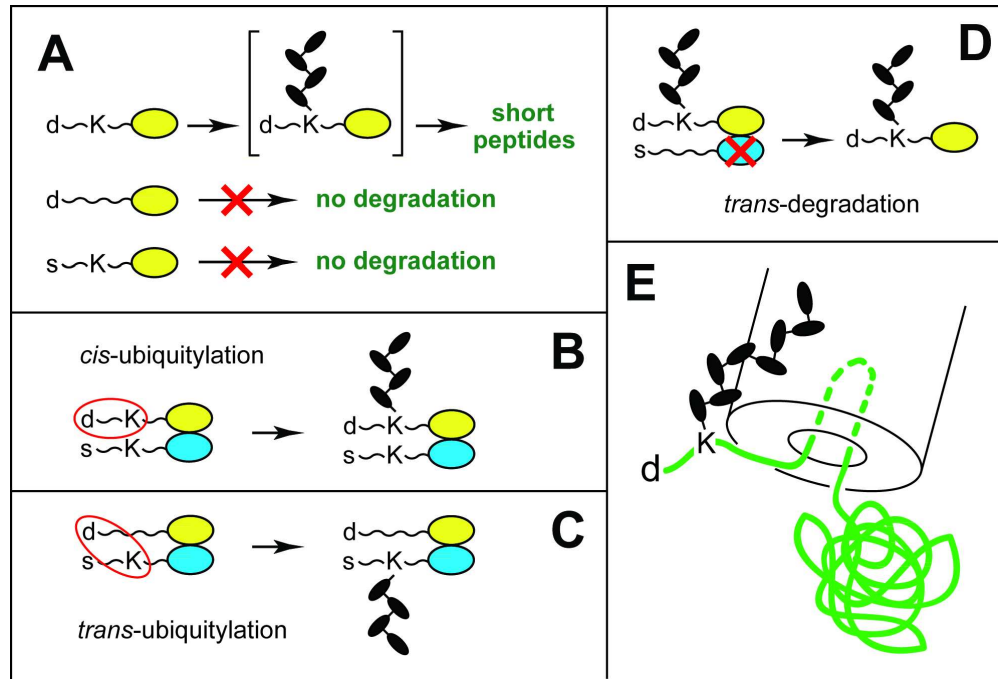


Fig. 11, Varshavsky

Fig 11_Varshavsky_PRO_11-0142
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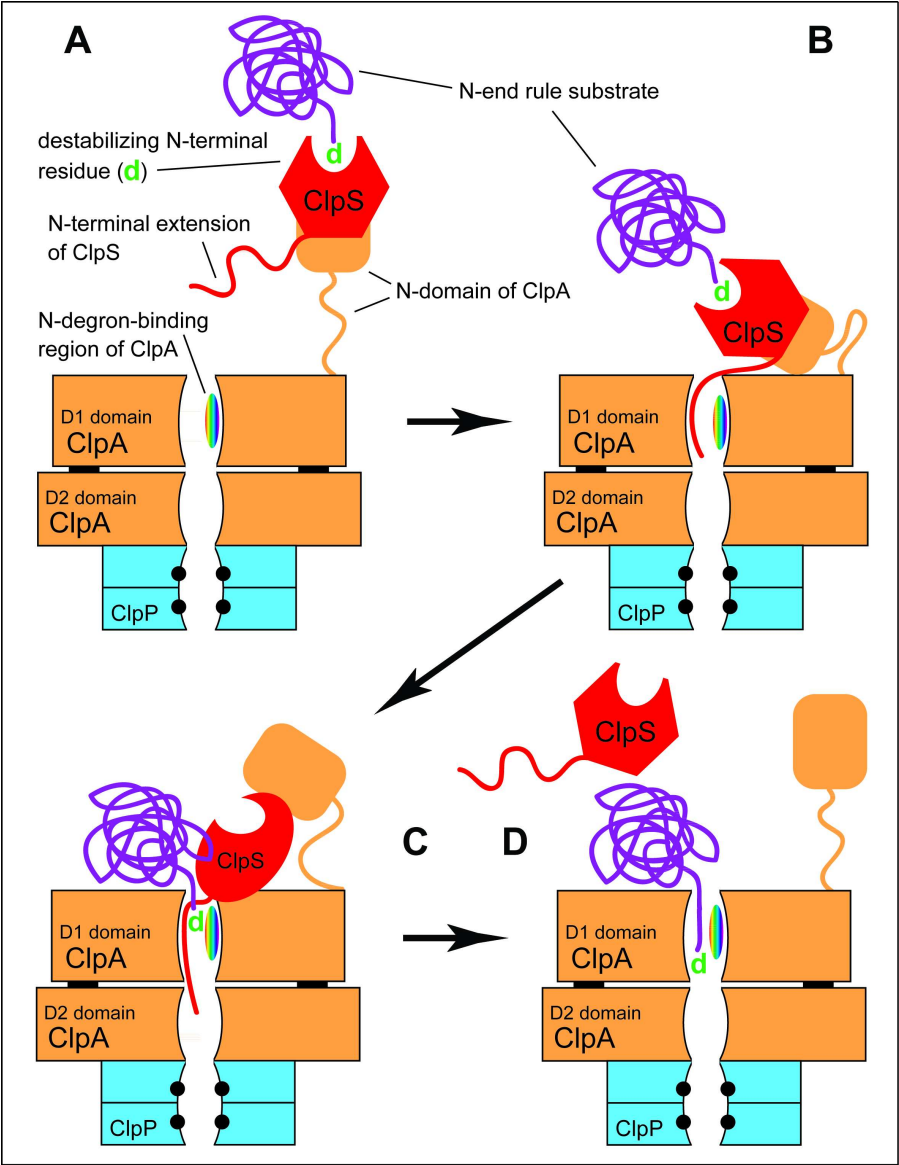


Fig. 12, Varshavsky

Fig 12_Varshavsky_PRO_11-0142
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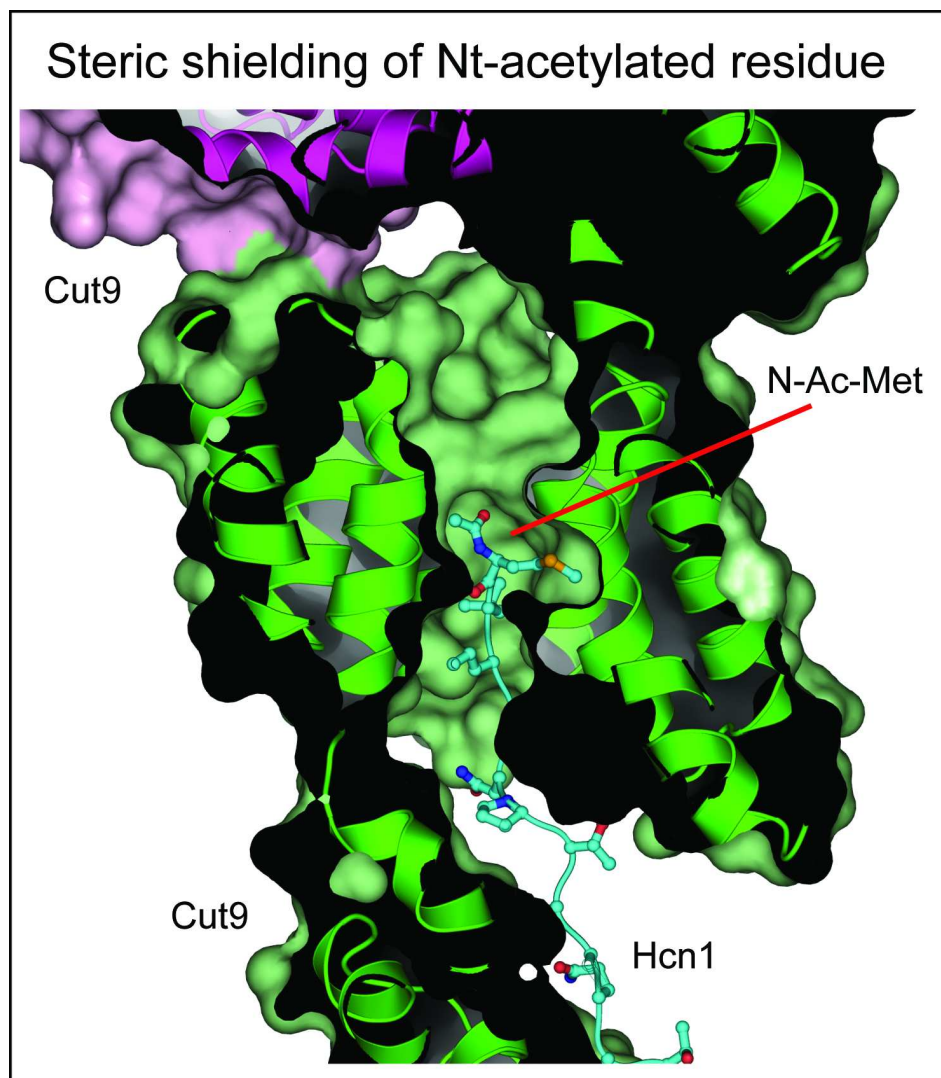


Fig. 13, Varshavsky

Fig 13_Varshavsky_PRO_11-0142
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Ntaq1 Nt^Q-amidase vs. transglutaminase

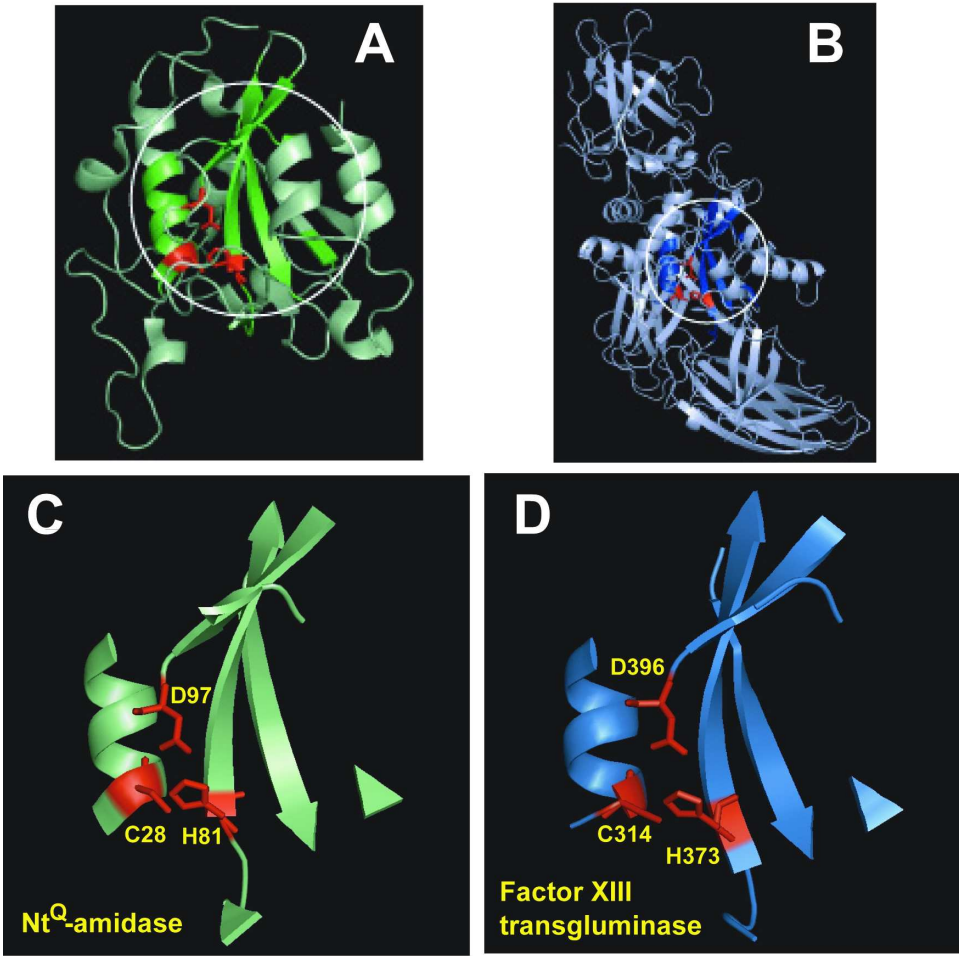


Fig. 14, Varshavsky

Fig 14_Varshavsky_PRO_11-0142
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Regulation of peptide transport by the Arg/N-end rule pathway

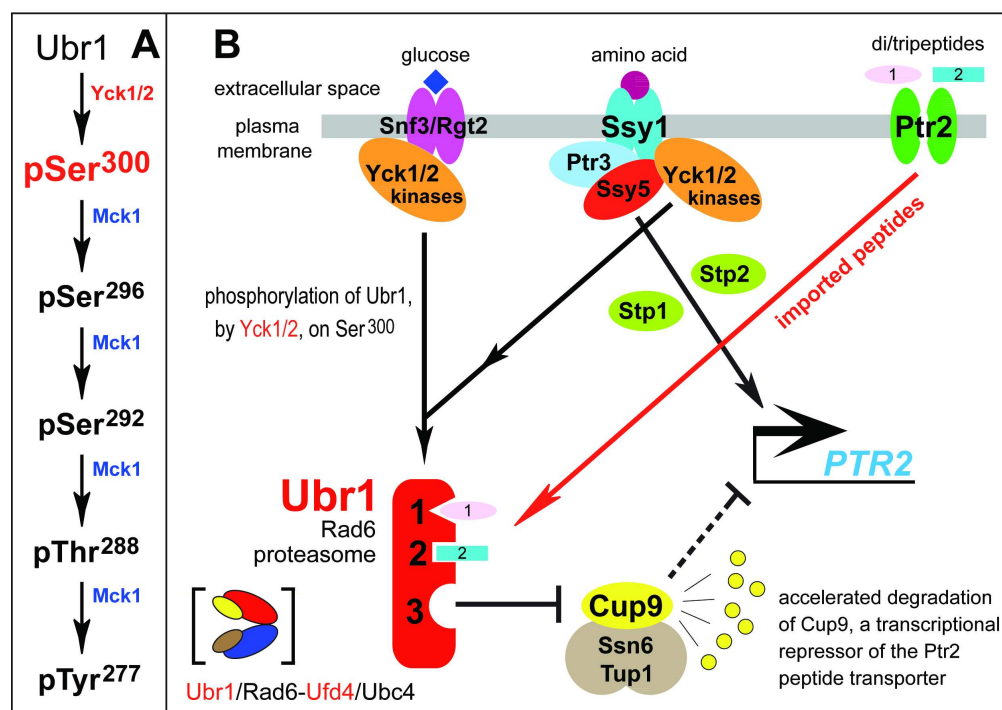


Fig. 15, Varshavsky

Fig 15_Varshavsky_PRO_11-0142
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